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ROUTINE EXAMINATION OF URINE FOR *MYCOBACTERIUM TUBERCULOSIS.*

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ALTHOUGH numerous different techniques have been proposed for finding acidfast bacilli in urine (Hallinan and Reusser, 1941 ; Kelso and Galbraith, 1943), none has received wider acceptance than that developed by Petroff. His procedure, described originally by Brown (1915), embodies the concentration of bacteria in acidified urine through sedimentation with tannic acid, centrifugalization, and dissolving the sediment with acetic acid. A recent application of Petroff's method to a series of 443 specimens of urine from 107 suspected cases of renal tuberculosis has led to several modifications helpful to the routine detection of tubercle bacilli by means of smears and cultures of concentrated material.

METHODS.

Collection and Concentration of Urine.

Rubber-stoppered one-litre Baxter bottles, obtained from salvage, are cleaned, sterilized and prepared for issue by the addition of 1·5 ml. 1 per cent. acriflavine (3, 6 diamino, 10 methyl acridine chloride) and 40 ml. McIlvaine's buffer solution adjusted to pH 4·0, i.e. 24·6 ml. M/10 citric acid and 15·4 ml. M/5 disodium phosphate.

To each 24-hour specimen, chiefly the evening and morning voidings, add 5 ml. 5 per cent. tannic acid and refrigerate overnight.

With a suitable sterile pipette remove the precipitate, centrifuge at 3000 r.p.m. for 10 minutes, discard the supernatant fluid, add enough 3 per cent. sodium hydroxide to cover the sediment and digest it in a water bath or incubator at 37° C. for 20 to 30 minutes.

Add two or three volumes of physiological saline and centrifuge again. Decant the supernatant liquid, inoculate heavily at least two slopes of Petragnani's medium, and prepare smears in the usual way for staining by the Ziehl-Neelsen procedure. Smears of the concentrates adhere without the addition of serum or albumen.

All glassware and reagents must be sterile in order to control the mould menace which frequently accompanies the culturing of concentrates.

Culture Medium.

As prepared in this laboratory, Petragnani's medium has been found satisfactory for culturing *Mycobacterium tuberculosis* from all kinds of clinical specimens.

With continuous stirring, boil 150 ml. fresh milk and 100 g. finely-grated

potato for ten minutes. Cool to 60° C., add five whole eggs, and stir slowly until mixed evenly.

Add 12 ml. glycerol and 10 ml. 2 per cent. aqueous malachite green. Mix cautiously to avoid incorporation of air, filter through two thicknesses of gauze, and dispense approximately 8 ml. to culture tubes (1·5 × 15 cm.).

Plug with cotton; inspissate tubes in slanting position (Wallenstein, 1941) for 30 minutes. Cool slowly at room temperature and then refrigerate.

After inoculation of slants, protruding cotton-wool plugs are sheared off at the lip of the tubes; the remaining stub is pushed to at least one-half inch below the edge of the tube. Tubes are sealed with a paraffin-petroleum jelly mixture (2 : 1).

Colonies of *M. tuberculosis* usually appear after two weeks and within four weeks of incubation at 37° C. When no growth develops, final readings are made at the end of eight weeks of incubation.

RESULTS.

The method of collection and concentration has been applied to a series of 443 specimens of urine, of which 295 representative specimens were cultured on Petragnini's medium. Acidfast bacilli were found in 48 (10·8 per cent.) of the smears. Forty-nine (16·6 per cent.) of the concentrates cultured were positive for *M. tuberculosis*; however, six of the positive cultures originated with concentrates found negative by smear. Five positive concentrates were not cultured or were contaminated with mould.

Concentrates are suitable for guinea-pig inoculation. This was demonstrated by a series of inoculations with known positive and selected negative concentrates. One concentrate, negative by smear, was found to be positive by both culture and guinea-pig.

During the winter months less than 5 per cent. of specimens cultured were contaminated with mould. In spring and summer contaminants appeared in approximately 10 per cent. of specimens cultured. This incidence of contamination is considered low in view of the frequent rotations of laboratory personnel.

By collection of urine in the acriflavine-buffer solution the growth of other bacteria frequently present in specimens from cases of renal tuberculosis is inhibited. The concentration of acriflavine used exerts no effect upon *M. tuberculosis* (Petroff, 1921). With cultured concentrates it has been noted that the bacteriostatic effect of acriflavine appears to enhance the action of malachite green in Petragnani's medium.

CONCLUSIONS.

A routine method of detecting *M. tuberculosis* in urine by means of smears and cultures has been described. Results show that properly-prepared concentrates examined microscopically and culturally are far more valuable than microscopy alone. For routine work culture methods are often more feasible than animal inoculations.

The combination of collecting urine in an acriflavine-buffer solution, substituting sodium hydroxide for acetic acid in Petroff's method of concentrating urine and culturing on Petragnani's medium provided an effective and successful means of detecting *M. tuberculosis*.

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THE PHARMACOLOGY OF PATULIN.

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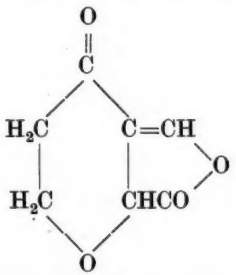
PATULIN is the name given by Birkinshaw *et al.*, 1943, to a substance obtained from *Penicillium patulum*, the composition of which is $C_7H_6O_4$, the structural formula being :



It appears to be identical with claviformin (Chain, Florey and Jennings, 1942, 1944) ; clavatin and clavacin (Wiesner, 1942 ; Waksman, Horning and Spencer, 1942, 1943 ; Anderson, 1943 ; Katzman, *et al.* 1944). As one of the substances produced by moulds with antibacterial activity, patulin appeared to be worth careful pharmacological investigation. The results of such a collaborative research carried out in the laboratories of the Member Firms of the Therapeutic Research Corporation of Great Britain and in the Department of Pharmacology, Oxford, are given below.

RESULTS.

1. *Toxicity.*

The LD50 for *mice* was found to be of the order of—

0·3	mg.	per	20 g.	given subcutaneously.
0·5	,,	,,	,,	intravenously.
0·7	,,	,,	,,	orally.

Intraperitoneally the toxic dose was of the same order as subcutaneously.

When death occurred very soon after intravenous injection it was preceded by convulsions. Port-mortem the lungs were found to be oedematous, fluid was found in the peritoneum and the liver was congested, these being signs of right-sided cardiac failure.

In those animals which died after oral administration of the drug pulmonary oedema was also a prominent feature.

The subcutaneous route was the most toxic. The site of injection showed inflammation, discoloration and oedema of the subcutaneous tissue. Gross visible signs included haemorrhagic kidneys, pale and flaccid spleens, oedema and haemorrhage in the lungs.

For *rats* the LD50 was 1·5 mg. per 100 g., which is equivalent to the dose for mice (0·3 mg. per 20 g.) after subcutaneous injection. The same inflammation, discoloration and oedema were found in the animals which died. The intravenous LD50 for rats was between 2·5 and 5 mg. per 100 g. ; convulsions preceded death. Five mg. per 100 g. caused increased secretion from the eyes and nose. Post-mortem pulmonary oedema was a common feature.

In *rabbits* which died after intravenous injection of patulin the main pathological condition was that of the lungs, which were oedematous, haemorrhagic and bathed in large quantities of fluid. In several rabbits the lungs were of the same colour as the liver.

In an unanaesthetized *cat* 5 mg. per kg. were given by intracardiac injection. This caused immediate salivation, the pupils were moderately dilated and the animal looked ill. Five days after the injection the animal was still not eating and its coat was staring ; the back and limbs were stiff. Post-mortem the liver was found to be heavily congested and dark in colour, the gall-bladder distended, the stomach and bowels empty and the lungs oedematous.

Chronic oral toxicity in mice.

A daily dose of 0·5 mg. per 20 g. killed about 50 per cent. of the mice within a fortnight. The mice dying during the actual experiment showed pulmonary oedema. Those killed at the end of the experiment showed pulmonary oedema to varying degrees. There was evidence of cumulative action.

Chronic experiments on mice using small doses subcutaneously and intraperitoneally.

Mice were injected with a 0·01 per cent. solution, the dose being 0·01 mg. per 20 g. for 11 days. All animals survived this period. Some were killed and examined on the day of the last dose ; the remaining animals were killed a week after the last dose. All animals injected subcutaneously showed hard patches and adhesions at the site of injection. These animals showed a slight gain in weight during dosing. The animals surviving a week after dosing showed considerable gain in weight. The animals injected intraperitoneally showed no microscopic lesions, but all these animals lost slightly in weight during dosing.

2. *Local Effects on the Skin.*

Subcutaneous injections into mice of relatively small doses of patulin (0·025 mg. per 20 g.) twice daily produced widespread subcutaneous oedema, which was very striking. Induration was noticed within an hour of the first injection and the injected area appeared tender. Rats and rabbits also showed considerable thickening of the skin and adhesions of the subcutaneous tissue. In rabbits intravenous injection into the ear vein produced oedema and hyperaemia. Perivenous injection caused ulceration and necrosis.

Doses of 0·25–1·0 mg. per 100 g. were injected subcutaneously into shaved areas on the backs of young guinea-pigs. Inflammation and necrosis varying in depth and extent with the size of the dose appeared at the site of injection 1–2 days later. The

reaction caused by larger doses persisted for at least a fortnight. Small daily doses of 0·01–0·05 mg. per 100 g. caused considerable necrosis after the first few doses, which persisted for at least 10 days after the dosing had ceased, eventually healing.

3. *Action on the Conjunctiva of the Rabbit.*

Patulin was instilled into the conjunctival sacs of three rabbits in strengths of 1 : 100, 1 : 1000 and 1 : 10,000. Within 15 minutes of administration rabbits I and II exhibited an inflammatory reaction of the conjunctiva, and in 2 hours marked chemosis was present. Within 24 hours the cornea in each case was obscured by a fairly dense haze apparently due to oedema. No solution of continuity in the corneal epithelium was noted after application of fluorescein. In rabbit III a slight conjunctivitis had also resolved and the eye appeared normal. On the other hand the affected eye in rabbit I exhibited a dense white opacity of the whole cornea. This had not improved 2 months after the experiment, and in addition there were large areas of abraded epithelium which gave the characteristic green staining with fluorescein. The animal was killed, the eye enucleated and dissected. The whole thickness of the cornea was found to be of uniform opacity. No conjunctivitis was present, or other macroscopical evidence of damage to the other ocular structures. (We are indebted to Dr. Wyers for his co-operation in this experiment.)

Thus patulin had a marked irritant action on the conjunctiva of the rabbit in a dilution of 1 : 1000. This concentration did not produce permanent damage, but a dilution of 1 : 100 caused abrasion of the epithelium and opacity of the cornea, which was still present after 2 months.

4. *The Effect of Patulin on Artificial Wounds.*

Eight guinea-pigs were depilated on both sides of the flank, and wounds of constant size induced by burning the skin with an electric iron under anaesthesia for a period of 5 seconds (Ungar and Robinson, 1944). After 5 days the scabs were removed, and four of the guinea-pigs' wounds were treated with patulin made up in the following ointment base :

Lanette wax . .	15 g.
Gum acacia . .	20 g.
Water to .. .	100 c.c.

This was melted with gentle heat and mixed with two dilutions of patulin to give a final concentration of 1 : 200 and 1 : 1000.

The left flank of the guinea-pigs was treated with patulin (ointment) 1 : 200, and the right flank with 1 : 1000. The patulin ointment was freshly prepared on alternate days. Four control guinea-pigs were treated with the ointment base only. About 10–12 days after treatment began the control wounds were healed, and the scabs came off, leaving a clean, glistening scar. But the two treated wounds had scabs still adherent with inflamed zones round them, and a visibly necrotic area in the peripheral parts of the wound. These scabs persisted for three weeks, and when removed showed deep ulcers penetrating to the abdominal wall.

Sections of wounds 9 days old treated with patulin 1 : 200 showed deep ulcers with leucocytic infiltration in the corium to the muscularis with remnants of muscle fibres. The ulcers were covered with a necrotic scab firmly adherent to the edges, with slight inflammatory changes in the surrounding corium. Similar changes with a more shallow ulcer were visible in the wounds treated with patulin 1 : 1000. Histological sections from wounds removed during the next 14 days showed gradual shallowing of the ulcer, which was filled with granulation tissue intermixed in the superficial zone

with small cell infiltration. After 21 days' treatment with patulin 1 : 1000, the wounds showed a thin layer of epidermis under the scab covering the area of newly formed granulation, whereas wounds treated with patulin 1 : 200 still showed a shallow ulcer, which was unaltered in the 28 days old sections.

These findings indicate that patulin in a dilution of 1 : 1000, and more so, 1 : 200, delays the healing of artificial wounds in guinea-pig skin for a period of over a fortnight. Patulin in a dilution of 1 : 200 has, in addition, a necrotizing effect inhibiting the healing of wounds for a period of 4 weeks.

5. *The Effect of Patulin on Growing Chick Embryo Fibroblasts.*

Tests were performed to see the effect of increasing dilutions of patulin in a buffered solution at pH 6·2 added to growing cultures of chick embryo heart fibroblasts at 37° C., using the depression slide technique. The plasma, which, in addition to Tyrode's solution and embryonic extract, was added to the growing cultures, was buffered to pH 6·2. Tests performed on three different occasions have shown that over a period of 3 days no growth occurred with patulin in dilution up to 1 : 16,000. Growth appeared in cultures with dilutions of 1 : 32,000.

6. *Action on the Cilia.*

The activity of the cilia on the surface of the mucous membrane in the frog's mouth and oesophagus can be studied by measuring the time taken by a small piece of cork to travel a certain distance. The application of patulin, 1 in 10,000, slowed and gradually stopped ciliary activity, which did not recover. For example, in one experiment the average time for the first five readings was 70″ for a movement of 3 cm., for the second five readings 150″ ; the time then increased to more than 3 minutes, and very soon movements ceased altogether. When another piece of mucous membrane of the same frog was examined, which had been kept under the same conditions but not treated with patulin, the average time was found to be 80″ for 3 cm.

Observing the ciliary movement in the trachea of a cat anaesthetized with chloralose the average speed of movement of a piece of cork was found to be 4 cm. in 117″ (10 readings). After the application of patulin 1 in 10,000 for 2 minutes, no more ciliary movement could be detected ; it did not recover.

It is concluded that patulin 1 in 10,000 arrests ciliary movement.

7. *Action on the Circulation.*

(a) *Blood pressure.*

In decerebrate cats or in cats anaesthetized with chloralose, ether or nembutal, the intravenous injection of patulin in doses from 4 to 8 mg. caused a rise in blood pressure followed by a fall. The rise was brief, like that due to the injection of adrenaline, while the fall was prolonged, and in part persisted (see Fig. 1*a*). Successive injections resulted in a steady fall in blood pressure. The effect was less harmful if the injections were made intra-arterially (see Fig. 1*b*), which suggested that a sudden high concentration arriving at the right side of the heart might produce a bad effect either on the heart or on the lungs.

The rise of blood pressure produced by intravenous or intra-arterial injection of patulin was not affected by ligature of the vessels to and from the suprarenal glands (see Fig. 1*c*). The rise was, however, abolished by full doses of ergotoxine, which abolished the pressor action of adrenaline (see Fig. 1*d*).

If adrenaline was slowly infused into a vein to maintain a constant level of higher blood pressure, the injection of patulin caused no rise, but only a fall in blood pressure which might pass off (see Fig. 2). Patulin reduced the pressor effect of adrenaline (see Fig. 3).

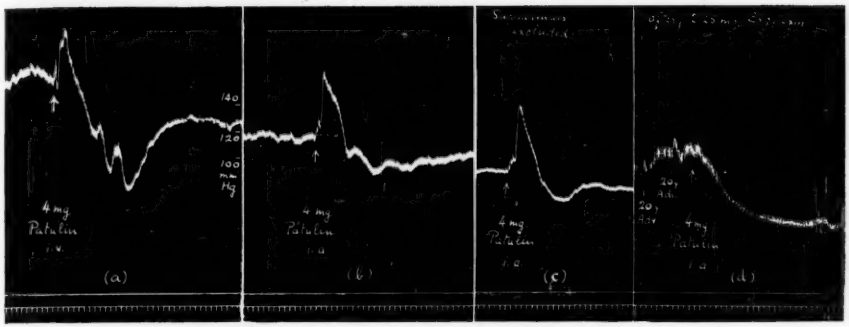


FIG. 1.—Cat, chloralose, blood pressure record. (*a*) 4 mg. patulin i.v., (*b*) i.a., (*c*) after ligature of the suprarenal vessels, (*d*) after ergotoxin.

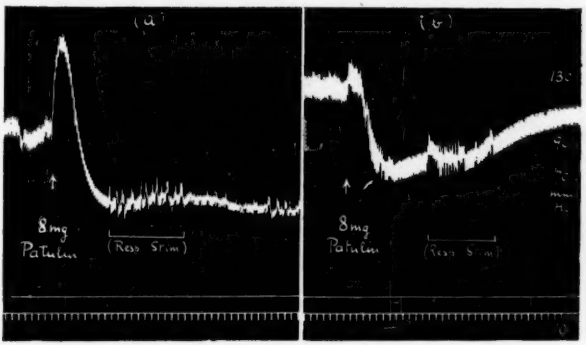


FIG. 2.—Decerebrate cat; vagi cut; artificial respiration. (*a*) 8 mg. patulin before, (*b*) during an adrenaline infusion.

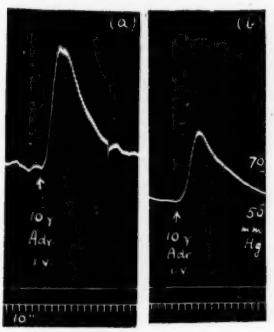


FIG. 3.—Spinal cat. The pressor effect of 10 μg. adrenaline (*a*) before, (*b*) after 16 mg. patulin.

On the blood pressure of the spinal cat patulin had no pressor effect, and if the blood pressure was already low not much depressor effect. The fall in blood pressure was also observed in the eviscerated cat. It was not abolished by atropine.

In rabbits anaesthetized with urethane and in dogs anaesthetized with chloralose the main effect of patulin was a fall in blood pressure which, after large doses, did not recover.

(b) *Action on the heart.*

In the whole animal, while the blood pressure was raised, the heart rate was often

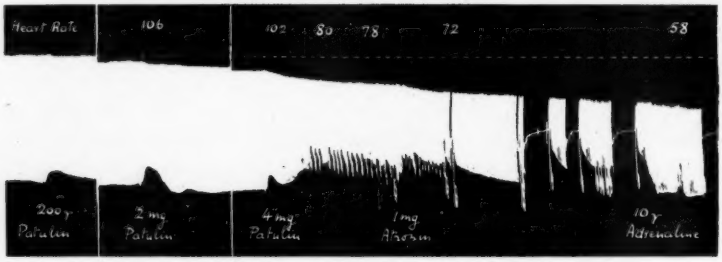


FIG. 4.—Rabbit, isolated heart. Note the inhibitor effect of patulin, which is irreversible and is not antagonized by atropine or adrenaline. The heart stopped in systole (downward movement).

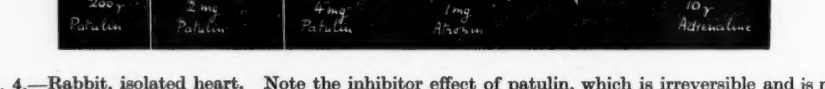


FIG. 5.—Rabbit's ear perfusion. 80 μg. patulin has a slight constrictor, 150 μg. a small but prolonged dilator action. The constrictor action of 0·01 μg. adrenaline is much reduced.

accelerated. When the fall of blood pressure had taken place the heart rate was slowed. Patulin had almost no effect on the left auricular pressure.

On the isolated perfused rabbit's heart (Langendorff's preparation) doses of 0·05–1·0 mg. patulin caused a transient weakening of the heart beat, with a slowing of the rate and an increase in coronary flow. 2 mg. patulin caused a more marked depression. Larger doses did not have a much greater immediate effect, the depression being only more prolonged. After 4 mg. the heart beat became very slow and weak. Atropine had no effect, nor had adrenaline. The contractions of the heart became irregular, diastolic relaxation was incomplete, and finally the heart stopped almost in systolic contraction (see Fig. 4).

The isolated frog heart (Clark's preparation) also stopped in systolic contracture on perfusion with concentrations of patulin of 10^{-3} and $0{\cdot}5 \times 10^{-3}$.

In the isolated frog heart (Straub's method) patulin 10^{-4} sometimes decreased the amplitude and abolished the response to adrenaline. Patulin 10^{-3} and 10^{-2} had a marked depressant effect.

(c) *Action on blood vessels.*

On the vessels of the dog's hind legs perfused with defibrinated blood doses of patulin from 0·1–5·0 mg. caused vasodilatation, shown by a fall in perfusion pressure and increased venous outflow. During an adrenaline tone this vasodilatation was more transient, and it was preceded by a very slight reduction in venous outflow indicating some preliminary vasoconstriction.

On the vessels of the rabbit's ear perfused with Ringer's solution (Gaddum and Kwiatkowski, 1938) 0·08 mg. patulin (the smallest dose having any effect) caused very small, transient vaso-constriction. Doses from 0·5–2·0 mg. caused prolonged vasodilatation. The vaso-constrictor effect of adrenaline was much reduced after the administration of patulin (see Fig. 5).

The vessels of the frog's hind legs were perfused with Ringer's solution containing 0·25 per cent. gelatine using a method described by Hijman and Chambers (1943), by which the gradual increase in weight due to the slow onset of oedema is recorded. The rate of oedema formation is indicated by the slope of the tracing. On perfusion with 1 in 10,000 patulin there was sometimes vasoconstriction shown by a fall in weight of the preparation when patulin first entered the vessels. This slight vaso-constriction was transient and not always present. Sometimes it was followed by an increased rate of oedema formation, which was the only effect seen with a solution 1 in 5000 (see Fig. 6).

(d) *Action on the spleen volume.*

In the cat anaesthetized with chloralose the injection of patulin (4–8 mg.) caused a large contraction of the spleen coincident with the rise of blood pressure. If, however, both splanchnic nerves were cut at a point where they pass underneath the diaphragm, then patulin caused no rise in blood pressure and no contraction of the spleen (see Fig. 7). In spinal cats, as already mentioned, patulin caused no rise in blood pressure. Neither did it produce a change in spleen volume.

(e) *Conclusion.*

It is therefore concluded that the pressor effect of patulin in the whole animal is due to a central action on the medulla prevented by division of the spinal cord or by section of the splanchnic nerves. The peripheral action of patulin is mainly vaso-dilator, occasionally preceded by a very transient vasoconstriction. Patulin has only little action on the heart.

8. *Action on the Respiration.*

In the decerebrate or chloralosed cat patulin (4–8 mg.) stimulated respiration. This stimulation was observed after cutting the vagi, and it also appeared if the animal was respired artificially to ensure adequate oxygenation (see Figs. 2 and 8). It was not wholly due to the fall of blood pressure.

In rabbits anaesthetized with urethane a large dose of 20 mg. patulin caused a fall both in rate and depth of respiration.

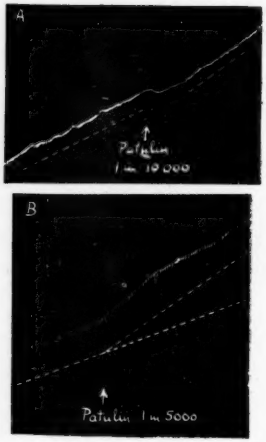


FIG. 6.—Frog's hind leg perfusion. During patulin 1 in 10,000, (*a*) after a transient vasoconstriction the slope of the tracing becomes steeper, indicating increased rate of oedema formation; (*b*) with 1 in 5000 this effect is much moro marked.

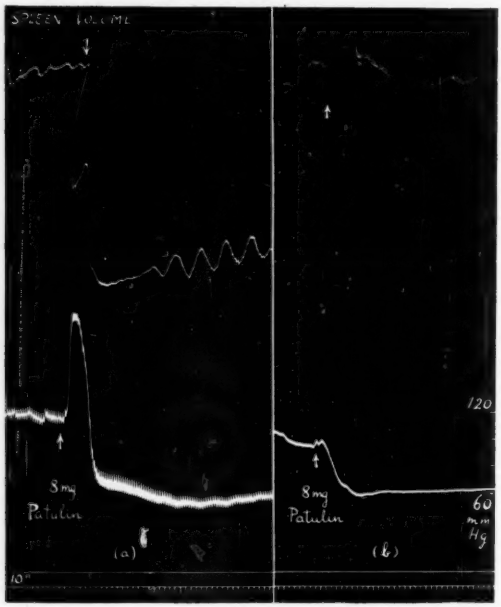


FIG. 7.—Decerebrate cat. The effect of 8 mg. patulin on spleen volume and blood pressure. (*a*) Before, (*b*) after cutting both splanchnic nerves.

9. *Action on the Medulla.*

Evidence was obtained of a stimulant action of patulin on the vasomotor centre as well as on the respiratory centre. In order to see whether patulin affected the

vomiting centre, pigeons were injected intravenously, but doses from 0·5 to 5·0 were without effect.

10. *Antidiuretic Action.*

Patulin was found to have a marked antidiuretic effect. Rats were given 5 c.c. warm tap water per 100 g. by stomach-tube. Patulin was given orally, subcutaneously and intraperitoneally. The observation period was 4 hours. The effects are shown in Table I, in which it is seen that the greatest action was exerted when patulin was injected subcutaneously. As little as one-fiftieth of the LD50 produced an antidiuretic action, and 0·25 mg. per 100 g., which is one-sixth of the lethal dose, almost abolished diuresis.

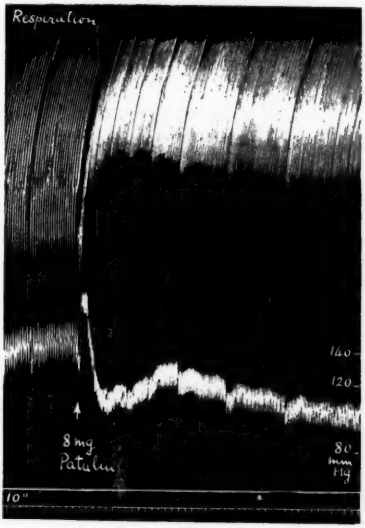


FIG. 8.—Decerebrate cat. Effect of 8 mg. patulin on respiration and blood pressure.

TABLE I.

Dose given: mg./100 g.	Orally, per cent. water excreted.	Subcutaneously, per cent. water excreted.	Intraperitoneally, per cent. water excreted.
None	65·6	68·4	66·5
0·031	..	50·6	..
0·062	..	42·0	60·2
0·125	68·0	31·4	56·2
0·25	66·0	7·1	3·9
0·5	51·7	3·4	..
1·0	31·5	..	..
2·5	0	..	..
5·0	0	..	..

11. *Action on Smooth Muscle.*

In the isolated rabbit's intestine suspended in a bath of 50 c.c. doses of less than 1 mg. patulin reduced pendulum movements and longitudinal movements and

diminished the tonus. Larger doses (1 to 5 mg.) gradually stopped all activity. This arrest was irreversible. On the guinea-pig ileum small doses of patulin which had no effect themselves abolished the responses to histamine, acetylcholine and also to barium chloride. Concentrations of 1 in 40,000 completely inhibited the spontaneous contractions.

The isolated rabbit's uterus was relaxed by patulin in concentrations from 1 in 50,000. This abolished spontaneous tone as well as that produced by adrenaline.

A concentration of 1 in 40,000 depressed the response of the isolated guinea-pig uterus to histamine. This depression remained after washing out.

12. *Action on Blood Constituents.*

(a) *Haemoglobin and red cell count.*

In rabbits 5 mg. per kg. caused a slight fall in haemoglobin concentration, while doses of 10 and 20 mg. per kg. caused an increase up to 32 per cent. above the original level, which was highest 5–8 hours after the administration.

In rats even 0·125 mg. per 100 g. (less than one-tenth of the LD50) caused considerable haemoconcentration, which was very marked with 2 mg. per 100 g. In some rats the haemoglobin level rose to over 200 per cent. of the original level. In all cases the blood was very dark and extremely viscous. It was found that while the haemoglobin level rose considerably, the red cell count rose only very slightly. As a number of " ghost cells " were noticed while counting the red cells, patulin was added to human blood *in vitro* ; the blood was incubated for 18 hours at 37° C., and the initial red cell count was compared with that after 3 and after 18 hours ; a deleterious effect was observed as shown in Table II.

TABLE II.

Patulin dilutions.	Red cell counts. Millions per c.mm. 0.	3 hours.	18 hours.
1 : 125	5·24	3·00*	0·67*
1 : 250	5·85	5·38	4·69
1 : 500	5·90	5·60	5·55

* Ghost cells.

(We are indebted to Mr. W. Freeman for the results given in Table II.)

The oxygen saturation curve of haemoglobin was definitely shifted after the addition of patulin as shown in Table III.

TABLE III.

(*a*) Normal rabbit blood. Partial pressure of O_2.	Percentage HbO_2 (approx. figures).	(*b*) Rabbit blood containing 0·05 per cent. patulin. Partial pressure of O_2	Percentage HbO_2 (approx. figures).
		1·8 mm.	10 per cent.
		Patulin added	
1·5 mm.	5 per cent.	1·8 mm.	0 per cent.
3·7 ,,	10 ,,	5·8 ,,	0 ,,
9·7 ,,	20 ,,	13·8 ,,	10 ,,
21·2 ,,	65 ,,	25·8 ,,	30 ,,
29·6 ,,	80 ,,	41·4 ,,	65 ,,
37·8 ,,	90 ,,	59·8 ,,	85 ,,
60·0 ,,	100 ,,	79·8 ,,	95 ,,
...	..	89·8 ,,	100 ,,

(b) *Blood sugar.*

In rabbits patulin was found to cause a rise in blood sugar. A dose of 5 mg. per kg. caused an average rise to 115 per cent. of the original level. After 10 mg. the blood sugar rose to 130 per cent. of normal within an hour and then returned to the initial level. After 20 mg. per kg. rises in blood sugar to 150–200 per cent. of normal were observed. Sometimes the rise continued for 5 hours, and the high level was maintained for at least 8 hours. Of the 11 animals receiving this dose four died within 24 hours, six died within 48 hours, and only one survived. This rise is probably due to a central action.

(c) *Blood urea and chlorides.*

In rabbits which received 4·0 and 8·0 mg./kg. patulin subcutaneously, doses which are at the toxic level, there was some increase in blood urea and a decrease in blood chlorides.

(d) *Alkali reserve of the blood.*

Rabbits were used. Determinations were carried out in duplicate on oxalated heart blood under paraffin.

Table IV shows that there was a marked fall in the alkali reserve of the plasma. The fall in CO_2 capacity is, however, reversible, as recovery occurred within 24 hours.

TABLE IV.—*CO_2 Capacity of Plasma (volumes per cent.) in Rabbits Injected Intravenously with Patulin 5 mg./kg.*

	Controls.	Rabbits receiving patulin.
1st experiment	47·8	28·4 (after 3 hours).
2nd experiment	41·2	38·0 (initially).
	33·2	19·5 (after 3 hours).
3rd experiment	40·9	28·5 (initially).
	38·9 (after 5 hours)	16·9 (after 5 hours).
	38·0 (after 24 hours)	30·3 (after 24 hours).

13. *Determination of Patulin.*

Although patulin is not a quinone, it was found that a colour reaction described by Craven (1931) generally applicable for quinones with a labile hydrogen or halogen atom adjacent to the carbonyl group could be used for its estimation. It consists of an extraction with ethyl acetate, followed by the addition of alcoholic ammonia and ethyl cyano-acetate. A red colour is produced which develops to a maximum in about 3 hours, and is stable for at least two days. This reaction was found to be quantitative over a range of 0 to 5 mg. of patulin. Higher concentrations were not investigated. The colour can be measured photometrically with the use of a filter with maximum transmission at about 5300 A.U.

This reaction has not been investigated sufficiently to state its specificity for patulin as such; the quantitative reaction observed may possibly be due to decomposition products.

(a) *Recovery of patulin from solutions in blood.*

An experiment was carried out to determine the percentage recovery of patulin from ox blood *in vitro*, after standing at room temperature for varying periods, com-

pared with recovery from solutions in water. Table V shows the results obtained. Solutions of 5 mg. in 5 c.c. blood were used.

After 16 hours, hardly any of the compound was present.

It was shown here that, by some process, patulin is changed in the blood to a form undetectable by the method used.

TABLE V.—*Percentage Recovery from Blood.*

Time.	0 min., per cent.	15 min., per cent.	30 min., per cent.	45 min., per cent.	60 min., per cent.
1st experiment	100	100	78	60	52
2nd experiment	100	76	60	56	..

Patulin was added to phosphate buffers of pH 7·0 and pH 7·4, to water and to blood, and the solutions were extracted at intervals of ¼, 1, 7 and 24 hours after the patulin had been added. Solutions consisted of 1 mg. in 5 c.c., and were kept at room temperature with occasional shaking.

Extractions were carried out as follows :

5 c.c. of solution were diluted with 10 c.c. water (this is necessary to haemolyze blood before extraction), and extracted with 3 × 25 c.c. portions of ethyl acetate in a separating funnel. The combined extract was washed with 10 c.c. water, and concentrated by boiling to a final volume of 5 c.c., to which was added at intervals of 10 min. 8 c.c. of alcoholic ammonia and 0·1 c.c. ethyl cyanoacetate. The colour which developed after 3 hours was measured photometrically, and the amount of patulin present determined from a curve prepared from solutions in water extracted immediately after the addition of patulin. The recoveries are shown in Table VI.

It appears from these experiments that the disappearance of patulin as such from blood is due to causes other than pH. The possibility was considered of enzymic action. Accordingly solutions of patulin were prepared in blood and water containing 0·1 per cent. potassium cyanide, but when extraction was carried out a few hours later no colour was developed in either solution. Similar solutions were prepared containing trichloracetic acid (2·0 per cent.), and after 24 hours' standing this was found not to affect significantly the disappearance of patulin from the blood.

TABLE VI.

Age of solution.	Percentage recovery. ¼ hr.	1 hr.	7 hrs.	24 hrs.
Solutions in :				
Water	60	60	..	50
Phosphate buffer pH 7·0	30	..	60	45
,, ,, pH 7·4	65	80	15	..
Blood (oxalated)	8·0	5·0	1·0	..
Blood with trichloracetic acid	..	..	..	1·0
Water with trichloracetic acid	..	..	..	60

During the process of extraction the blood is haemolysed, so that an absorption phenomenon is discounted. The velocity of the reaction tends to discount simple physical absorption or adsorption, and suggests an enzymic reaction, particularly on account of the large amount of the compound taken up by the blood.

(b) *Blood concentration and urinary excretion in the rabbit.*

Although good recovery can be obtained from blood *in vitro*, the recovery falls off fairly quickly when the drug is allowed to remain in contact with the blood for some time. Therefore it has not been possible to detect patulin in any measurable amount in the blood of injected animals ; and in the urine also only 5 per cent. of the amount given could be recovered within 48 hours.

SUMMARY.

The most striking pathological change produced by lethal doses of patulin is severe lung oedema. Patulin also causes oedema when small doses are injected subcutaneously ; this oedema is not confined to the site of injection, but occurs throughout the body. This leads to haemoconcentration. The capillary damage can also be observed in isolated perfused organs where patulin increases the permeability of the dilated blood vessels and thus increases the rate of oedema formation.

Patulin has a local irritant action on the conjunctiva, it arrests the ciliary movement on mucous membranes, it stops the growth of fibroblasts in tissue cultures and inhibits wound healing. These effects suggest that patulin is a tissue poison.

Another interesting observation is the marked antidiuretic action of subtoxic doses of patulin. With as little as one-fiftieth of the LD50 an inhibition of water diuresis is observed.

Patulin has a stimulant action on the respiratory and vasomotor centres in the medulla. It causes a rise in blood pressure by this central action, which is followed by a fall in blood pressure due to the dilator action of patulin on the vessels themselves. Patulin relaxes all smooth muscle.

Patulin causes a rise of blood sugar, which is probably also a central medullary effect.

Patulin is considered to be too toxic to be of therapeutic use.

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AN ANTIBIOTIC FROM *CREPIS TARAXACIFOLIA* (THUILL.).

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RECENT work on antibiotics formed by bacteria and moulds (Waksman, 1941; Chain and Florey, 1944) has stimulated interest in other naturally occurring antibacterial substances. Osborn (1943) has shown that watery extracts of many species of plants possess bactericidal or bacteriostatic properties. In several cases the plant exhibiting such action was known to contain or to give rise to, on crushing, a bacterial inhibitor such as cyanide, a polyphenol, quinone, saponin, isothiocyanate, etc., but in others no such clue could be found in the literature as to the probable nature of the inhibitor. *Crepis taraxacifolia* (Thuill.) was one of these latter, and the isolation in the crystalline state of the inhibitor is described below. For convenience the name " crepin " will be used in the present account to designate this substance.

In England this plant occurs in fields, banks and waysides. Flowering may extend from April till October, reaching its peak during May and June. Watery extracts of the buds, flowers, inflorescence stems and roots were found by Osborn (1943) to contain a substance inhibiting *Staphylococcus aureus*; it was also shown that drying of the plant did not abolish this property. The inhibitor did not occur in the plant in the free form, but as an antibiotically inactive precursor, since an extract of steamed material which had no inhibitory power acquired full potency when incubated for a short time with a small quantity of raw extract. The agent—presumably an enzyme—causing the activation of the inactive precursor appeared to be confined to the yellow petals of the flower, and, to a much smaller extent, to the root; the substrate, from which it liberated the inhibitor (crepin), occurred in most parts of the plant except the stem and leaves; the seed contained neither enzyme nor substrate (Osborn, unpublished observations). The closely related *Crepis virens* behaves in a similar way, and though no proof was obtained, it seems probable that here the inhibitor is identical with or very similar to crepin.

Assay.

The cylinder-plate method, as developed for penicillin (Abraham *et al.*, 1941; Heatley, 1944), was used throughout the present work, *Staph. aureus* being the test organism. Since pure crepin is only slightly soluble in water, a zone of inhibition greater than 17–20 mm. in diameter is seldom obtained; super-saturation does, however, occur, and the fact that zones up to 25 mm. were occasionally obtained caused the low solubility to be overlooked for some time. The early fractionation experiments were therefore sometimes difficult to understand and misleading conclusions were drawn from them.

Extraction of inhibitory substance.

Many different methods of extraction were tried, starting with aqueous, alcoholic or chloroform extracts, and applying chromatographic adsorption, molecular distillation and other techniques of separation to them. The method finally adopted was as follows:

About 250 g. of fresh, or 50 g. of dried flowers and buds are ground in the mechanical mortar with 100–150 ml. of distilled water and about 50 g. of sand. The pulp is squeezed in linen in a press, then kneaded with a further 100–150 ml. of water and again pressed out. The pooled dark brown cloudy, watery extracts are acidified to pH 2·5–3·0 with hydrochloric acid, and the resulting dark greenish-brown flocculent precipitate is centrifuged down. This precipitate is stirred with 50–100 ml. of N/100 hydrochloric acid and again centrifuged, then discarded. The centrifugates, which are quite clear and the colour of weak tea, are passed through a bed about 1 cm. thick of a mixture of 5 g. of Celite or kieselguhr and 15 g. of Farnell grade 14 activated charcoal—the more dilute extracts being passed through first. (This technique and this particular grade of charcoal was suggested, in another connection, by Dr. C. G. Pope.) The bed is then washed with a small quantity of water. The first colourless filtrates are discarded, but the later portions and the washing (which may all contain small amounts of unadsorbed crepin and are very faintly coloured), are passed through the new charcoal bed of the next batch. The crepin is then eluted from the charcoal by sucking 80 per cent. aqueous acetone very slowly through the bed, which has not been disturbed in any way. The first percolates are strongly pigmented, but the elution is continued till the emerging solution is colourless (again the final percolates are not worked up directly, but are used for the first elution of the charcoal bed of the following batch). From the richest eluates (in which, on pooling, a whitish precipitate usually separates out) the acetone and some of the water are removed by distillation, first at normal, then under reduced pressure. The pH of the resulting solution is adjusted to 6·0–6·5, and it is extracted, together with any precipitate, with ether in a liquid-liquid extractor for 16–24 hours. The ethereal extract is passed through a short column of alumina, which removes a small amount of green pigment, then it is concentrated and placed in the refrigerator; crystals of crepin appear on the walls of the vessel after some hours. On further concentration a second crop may be obtained, but at this and later stages a very bitter gummy mass separates with the crystals and is difficult to remove from them. The crude solid may be recrystallized from alcohol or alcohol and water. The yield is variable and small, rarely exceeding 100 mg. of pure crystals per kilogram of fresh starting material.

Chemical and physical properties.

Crepin crystallizes from absolute and 50 per cent. alcohol in a variety of crystalline forms. (See note by Rogers following this paper.) It is odourless and tasteless, though all but the purest samples have a bitter taste. It is very soluble in pyridine, soluble in water at room temperature to the extent of about 0·25 mg. per ml., and about ten times as soluble as this in alcohol. It is also soluble in ether and glycerol; if a drop of the latter solution is left exposed on a slide, crepin will separate out in the form of very thin plates, as moisture is taken up from the atmosphere by the glycerol.

Crepin can be heated to 300° C. without melting, though considerable darkening occurs at this high temperature. On heating strongly it chars completely, giving off dense yellowish-white fumes which condense to a tar on the cooler parts of the tube.

Nitrogen, halogens, sulphur, phosphorus and ash are absent. Elementary analysis (Weiler and Strauss) gave C 68·3 per cent., H 6·4 per cent. on one sample, and C 68·4 per cent., H 6·3 per cent. on a second, which had been prepared a year later. The formula $C_7H_8O_2$ would have C 67·8 per cent., H 6·4 per cent. As the material is insoluble in camphor a Rast molecular weight determination could not be made, but by the method of Barger (1904) the molecular weight was found to lie between 236 and 275. The evidence suggests the molecular formula $C_{14}H_{16}O_4$, which has a molecular weight of 248.

The aqueous solution of crepin has a neutral reaction and does not decompose sodium carbonate solution, so carboxyl groups are absent. Weiler and Strauss found O-Me absent, but 1·02 equivalents of C-Me per molecule (taking the formula as $C_{14}H_{16}O_4$). Ammoniacal silver nitrate and Fehling's solution are reduced to a negligible extent, and no precipitate is formed with 2·4 dinitrophenylhydrazine. No colour is given with ferric chloride, but an estimation of hydroxyl groups by the method of Freed and Wynne (1936) gave values of 1·66 and 1·64 groups per molecule. Iodine is not liberated from acidified potassium iodide. Both potassium permanganate and bromine water are decolorized, the latter with simultaneous formation of a white opalescent precipitate and the uptake of 4 atoms of bromine per molecule. Under catalytic hydrogenation (with palladium-on-charcoal as catalyst) 4 atoms of hydrogen per molecule are readily taken up, the antibiotic activity being simultaneously destroyed.

The ultra-violet absorption of the material was kindly examined by Dr. Holiday, who reported the absence of any prominent absorption bands, and suggested that there could not be more than one pair of conjugated double bonds in the molecule.

When the substance is inactivated by treating with N/10 alkali at room temperature for 30 minutes, back titration reveals the appearance during inactivation of one titratable acidic group. Attempts to isolate an acid by acidification and extraction with ether were not successful, acidification resulting in a gelatinous precipitate which was insoluble in water or sodium carbonate, and in most organic solvents except pyridine, dioxane and glacial acetic acid. This substance could not be recrystallized, but elementary analysis after thorough washing suggested that it had the same formula as the original crepin from which it was obtained. (Found : C 67·7 per cent., H 6·2 per cent.—Weiler and Strauss.)

The test of Jacobs and Hoffmann (1926) for a β–γ unsaturated lactone was applied to crepin at Dr. E. P. Abraham's suggestion, and gave a definite positive reaction. (It is of interest in this connection that strophanthin, which also contains a β–γ unsaturated lactone group, had no appreciable antibiotic activity against *Staph. aureus*.)

So it appears that the opening, by alkali, of a hypothetical lactone ring in crepin is irreversible, although some other intramolecular condensation probably occurs.

Stability.

Crude plant extracts retained their activity for several weeks in the ice-chest. Experiments with such extracts and with purer material indicated that the activity was almost completely destroyed in 10 minutes at room temperature in the presence of N/10 alkali, and that it was also destroyed, but more slowly, at pH 9·0. It was, however, stable in acid solution, and could be autoclaved for 20 minutes at 15 lb. pressure in the presence of N/10 hydrochloric acid without noticeable loss of potency. Crepin was not liberated from the inactive form in which it occurs in the plant by this treatment.

Effect of crepin on bacteria.

The concentration of crepin which just inhibits the growth of *Staph. aureus* in a liquid medium depends on the number of organisms present, the titre approximately doubling as the number of bacteria in the inoculum is reduced one hundredfold. The titre also decreases as the time of incubation is prolonged ; e.g. with an inoculum of 50,000 organisms per ml. of Lemco broth the titre was 1 in 80,000 after 20 hours, 1 in 40,000 after 30 hours, and 1 in 30,000 after 48 hours. The inhibitory power towards a few other organisms was also tested in a semi-quantitative way, with the following results :

Staph aureus	1 in 32,000
Str. pyogenes	1 in 8,000
B. subtilis	1 in 16,000
Ps. pyocyanea	Less than 1 in 4,000
S. typhi	1 in 4,000

Staph. aureus was not lysed after 16 hours' incubation at 37° C. in a 1 in 4000 dilution of crepin.

Peptone was found to have no detectable effect on the titre, but human serum—either heated to 52° C. for 1 hour or untreated—had a definite action, as illustrated by the following experiment :

In a number of parallel dilution series of crepin in Lemco broth containing varying amounts of dried human serum, the titre towards *Staph. aureus* after 24 hours' incubation at 37° C. was as follows :

Control (no serum)	1 in 40,000
Containing the equivalent of 8 per cent. serum .	1 in 25,000
,, ,, 20 ,, ,, . .	1 in 15,000
,, ,, 50 ,, ,, . .	1 in 7,500
,, ,, 20 ,, heated serum .	1 in 15,000

This effect was shown *not* to be due to pH.

Effect on leucocytes.

This was examined by Dr. M. A. Jennings, who reported as follows :

"The effect on human leucocytes was examined by the method previously described (Abraham *et al.*, 1941), the leucocytes being in contact throughout the observations with a solution of the substance in special physiological saline.

"The highest dilution at which any effect was observed was 1 in 4,500,000, in which the cells became sluggish after an hour, and later stationary, though they survived as long as in the control preparations. In 1 in 450,000 and lower dilutions movement was arrested almost immediately, and soon some of the cells began to die. Nevertheless some survived, and even at 1 in 4500, the strongest solution tested, a few cells were alive after 2 hours.

"It appears remarkable that a substance which has some effect on locomotion at 1 in 4,500,000 should not kill all cells rapidly at a concentration a thousand times greater."

This work was carried out during the tenure of a grant from the Nuffield Provincial Hospitals Trust. I wish to thank Mrs. E. M. Osborn for botanical suggestions, and, with Misses M. Lancaster, P. McKegney and P. Morris, for help in collecting and working up material ; my thanks are also due to Dr. E. P. Abraham for chemical suggestions.

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A NOTE ON THE X-RAY CRYSTALLOGRAPHY OF CREPIN.

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CREPIN prepared as described in the preceding communication was obtained from two solvents, 50 per cent. alcohol and absolute alcohol, in three different crystallographic forms, A, B and C, the detailed characteristics of which are given below:

	A.	B.	C.
Solvent	50 per cent. alcohol	Absolute alcohol	Absolute or 50 per cent. alcohol
Description	Monoclinic tables	Orthorhombic prisms	Orthorhombic prisms terminated by pyramids
Optics	Plane of optic axes (010) γ approx. $//c$	$a//b$ $\beta//a$ $\gamma//c$	—
Space group	$P2_1$	$P2_12_12_1$	$P2_122_1$
Unit cell dimensions:			
a	5·93	12·37	12·4
b	7·91	5·12	9·1
c	22·95	21·15	11·8
β	38° 30′	—	—
ρ (density)	1·335	1·31	1·33
Molecular weight	270	266	268

The form A certainly contains solvent of crystallisation. Dr. Heatley's value of 6·41 per cent. corresponds well with the calculated value 6·76 per cent. for the formula $C_{14}H_{16}O_4.1H_2O$ (266).

Solvent of crystallization has not been observed in B or C. As they stand, the measurements on these two forms agree rather better with a molecular weight of 262 ($C_{15}H_{18}O_4$).

Calculated	C 68·7% ; H 6·87%
Observed (Weiler and Strauss).	C 68·4% ; H 6·3%

The relationship between the three forms has not been completely investigated. The monoclinic form was the first to be observed, and this was later obtained from solutions seeded with B and C.

STUDIES ON THE VIABILITY AND FILTERABILITY OF TYPHUS RICKETTSIAE.

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THESE studies were essential preliminaries to a more comprehensive programme of typhus research, and had as their objective the provision of data to form the basis of a reliable experimental procedure for later work.

The rapidity with which typhus rickettsiae normally lose their viability when suspended in relatively simple liquid media is well known. Our attention, therefore, was first directed towards determining the conditions most favourable for the retention of viability. The influences of the following factors were investigated: (1) the composition of the medium, (2) the pH of the medium, and (3) the temperature. At least one other factor was suspected of affecting the activity of rickettsial suspensions, namely, the oxidation-reduction potential, but its investigation was deferred.

The early experiments were conducted with the murine (Wilmington) strain of typhus, grown both in egg embryos and mouse lungs. Initially crude rickettsial suspensions were employed, made by grinding the infected yolk sac or lung tissue with glass powder in the chosen liquid medium, generally phosphate saline. This was spun for 5 min. at 1500 r.p.m. and the supernatant fluid was used. Further, the early tests for infectivity were made by injecting serial dilutions of the fluid into the cleanly shaven skin of a rabbit and observing the reaction. This method was later discarded in favour of the mouse lung titration method (Andrewes, King, van den Ende and Walker (1944)). This consists in counting the discrete foci of infection which develop in the lungs of mice inoculated intranasally with 0·05 c.c. of serial dilutions of the rickettsial suspension.

Results of Viability Tests.

1. *The nature of the medium.*

A saline suspension of murine rickettsiae from mouse lung was concentrated by spinning for ½ hour at 12,000 r.p.m. and resuspending the deposit in one-fifth the original volume of supernatant. Then a 1 : 100 dilution of this suspension was made in the media indicated in Table I and kept at 0° C. Tests were made at chosen intervals, by the method of injection into rabbit's skin, of the undiluted and 1 : 10 serum broth diluted systems. The results are summarized in Table I.

Serum broth, of the media tested, clearly appears to provide conditions most favourable for the maintenance of viability of typhus rickettsiae.

TABLE I.—*The Viability of Typhus Rickettsiae in Various Media.*

Medium.	pH.	Period at 0° C. 2 hrs.		24 hrs.		48 hrs.		96 hrs.	
		Und.	×10.	Und.	×10.	Und.	×10.	Und.	×10.
Broth	7·69	++	+	±	tr.	tr.	—	—	—
Serum-broth	8·10	++	+	++	++	+	±	tr.	tr.
Saline	6·85	++	+	++	±	tr.	—	—	—
Serum-saline	7·93	++	+	++	+	tr.	—	—	—
Water	6·95	±	tr.	—	—	—	—	—	—
Peptone water	7·06	++	+	—	—	—	—	—	—
Tyrode solution	8·18	++	+	—	—	—	—	—	—
Supernatant fluid after spinning mouse lung suspension ½ hr. at 12,000 r.p.m.		++	+	+	±	—	—	—	—
Infectivity test on supernatant		—	—	—	—	—	—	—	—

2. *Influence of pH.*

A stock rickettsial suspension, from infected mouse lung, was prepared in serum broth at pH 7·6, by the procedure already described. This was diluted 1 : 100 in a series of phosphate, citrate and acetate buffers and tested at intervals while kept at 0° C. Results indicated the pH range for viability to be 5·5–7·5. We studied more recently, in collaboration with our colleague Dr. Fulton, the influence of pH on the viability of a suspension of murine rickettsiae purified by means of an adsorption technique (Fulton and Begg). We used the series of buffers described by Northrop and de Kruif (1921), making 1 : 100 dilution of the stock suspension in the buffers, and in serum broth as control. Tests were made after keeping for 1 hr. at room temperature, 20° C., and after 24 hrs. at 0° C. The "spot-count" method was used, 0·05 c.c. of a 50-fold dilution of each system being given intranasally to batches of four mice. The results are given in Table II.

TABLE II.—*The Influence of pH on the Viability of Typhus Rickettsiae.*

pH.	After 1 hour at 20° C. Spot counts.	Av.	After 24 hours at 0° C. Spot counts.	Av.
4·82	(d), 0, 0, 0	0	56, 63, 50, 62	56
5·78	77, 79, 56, 103	79	132, 112, 118, (d)	120
6·42	(25), 113, 92, 99	98	107, (o), (o), 88	97
7·18	122, 88, 87, 77	93	104, 98, (d), 120	107
7·61	(d), 116, 112, 100	109	134, 80, (35), (d)	107
8·20	++, 77, 90, 125	97	96, 85, 104, 106	98
8·82	(d), 54, 27, 54	45	92, 118, 121, (d)	111
9·35	(d), (0), 34, 23	28	(d), 76, 86, 87	83
Serum broth pH 7·6	88, 82, 100, 110	95	101, 86, 144, (d)	110

Key to Abbreviations used in Tables.

c = Gross consolidation of lung.
++ = Spots too numerous to estimate.
d = Died from non-specific cause.
Counts of 150 and over are approximate only.
The results in brackets were discarded in taking the average counts.

These figures confirm the earlier findings, but extend the zone of stability on the alkaline side. The optimum region appears to be pH 6 to 8·5.

3. *Influence of temperature.*

A suspension in serum broth of murine typhus rickettsiae from mouse lung was concentrated by centrifugation, and then fifty-fold dilutions were made in serum broth, pH 7·6, and kept at the following temperatures: 37° C., 23° C., 0° C., — 10° C., and — 77° C. Infectivity tests were made at intervals by injecting 0·05 c.c. amounts of the undiluted fluid and the 1/10 dilution into a rabbit's skin. Table III summarizes the results.

TABLE III.—*The Influence of Temperature on the Viability of Typhus Rickettsiae.*

Temperature.	Period elapsing before test.											
	24 hrs.		48 hrs.		4 days.		8 days.	
	Und.	×10.	Und.	×10.	Und.	×10.	Und.	×10.
37° C.	±	?	±	tr.	—	—	—	—
23° C.	tr.	—	—	—	—	—	—	—
0° C.	++	++	++	++	—	—	—	—
−10° C.	++	++	++	++	+	tr.	±	±
−77° C.	++	++	++	++	++	++	++	++

It appears, therefore, that at —77° C. typhus rickettsiae retain their viability unimpaired for many days in the medium here being used. More recent work (Andrewes, King, van den Ende and Walker (1944) has shown that even after four months at —77° C. typhus rickettsiae retain their viability. The better survival at 37° C. than at 23° C. is interesting, and would find explanation if some multiplication could occur in the presence of partially disrupted cellular tissue from the crude suspension used as the starting-point in these experiments. It is interesting to note here that Nigg (1935) found tissue cultures of typhus fever rickettsiae, kept under paraffin seal, could be preserved for months at 37° C. or —20° C., but survived for relatively short periods at 20° C. and —4° C.

Centrifugation Experiments.

These experiments were conducted to ascertain the conditions under which the centrifugation method might be used as a means of partially purifying and concentrating the suspensions of typhus rickettsiae.

(a) *Yolk sac suspension.*

One infected yolk sac, murine typhus, was ground up with Pyrex glass powder in 25 c.c. saline, and then spun for 2 minutes at 1000 r.p.m. to yield supernatant S1. This supernatant was divided into four portions, each of 5 c.c., and spun in the Ecco high-speed centrifuge under four different conditions. In each instance there was a surface layer of fat, the main bulk supernatant and a deposit. After removing the fat as completely as possible, the supernatant was withdrawn for test and the deposit taken up in 1 c.c. saline. Systems were kept in the ice chest at 0° C. until all were ready for test. The results are given in Table IV.

TABLE IV.—*Centrifugation of a Suspension in Saline of Typhus Rickettsiae from Infected Yolk Sac.*

	Centrifugal force, g.	Spot counts at indicated dilutions.				
		10.	10^2.	10^3.	10^4.	10^5.
Control supernatant S1	..	..	c	c	200–300	50–70
S1 after 15 min. at 6,000 r.p.m.	3200	..	c	++	200	..
,, ,, 7,000 ,,	4400	..	c	200–300	40–70	..
,, ,, 8,000 ,,	5800	c	300	20–40	10	..
,, ,, 10,000 ,,	9000	++	60–90	? 1	*nil*	..

(b) *Mouse lung suspension.*

The lungs of 14 mice infected with murine typhus were ground up with sand in 20 c.c. serum broth. This crude suspension was spun for 2 min. at 1000 r.p.m. to give supernatant S1, which was then subjected to the fractionation procedure under the conditions indicated in Table V.

TABLE V.—*Centrifugation of a Suspension in Serum-broth of Typhus Rickettsiae from Infected Mouse Lung.*

System.	Centrifugal force, g.	Spot counts at indicated dilutions.					
		10.	10^2.	10^3.	10^4.	10^5.	10^6.
Control supernatant S1	..	..	..	..	c	++	99, 109 80, 137
Supernatant S2—after spinning S1 for 15 min. at 2000 r.p.m.	350	..	..	..	c	++	107, 114 110, 116
Supernatant S3—after spinning S2 for 15 min. at 5000 r.p.m.	2200	..	..	..	c	d, d 200, 200	..
Supernatant S4—after spinning S3 for 15 min. at 10,000 r.p.m.	9000	..	++	85, 62 d, 70	..	..	..
Supernatant S5.—The deposit after spinning S3 was resuspended in Tyrode solution and respun at 10,000 r.p.m.	..	..	22, 46 19, 33	..	..	..	..

N.B.—The figures for the centrifugal force indicate the approximate value near the bottom of the tubes.

Other experiments with serum broth suspensions of lungs from mice infected with epidemic typhus indicated that spinning for ½ hr. at 7000 r.p.m. resulted in 90 per cent. of the rickettsiae being deposited.

The centrifugation figures were of much value in deciding upon our experimental procedure for obtaining relatively concentrated rickettsial suspensions freed from much of the tissue protein contained in the crude suspension.

Filtration Experiments.

Typhus rickettsiae are generally held to be non-filterable. Records in the literature show that only occasionally have rickettsiae been found to pass bacteriologically tested filter candles. Hence the conclusion that while rickettsiae are not to be classified

with the so-called " filterable agents " or viruses, yet, on the other hand, they appear smaller than the normal bacteria used for testing filters—e.g. *B. coli, B. prodigiosus.* Pinkerton (1942) has written " that the question of filterability needs further study under carefully controlled conditions. Isolated rickettsiae freed from remnants of cell cytoplasm and suspended in a medium in which they remained virulent for the duration of the experiment would have to be employed for this purpose." True, these are the ideal conditions one would like to obtain, but they are exceedingly difficult even to approach. Since typhus rickettsiae have, so far, not been adapted to growth on an artificial medium, we are forced, as already indicated, to use suspensions of ground-up infected tissue as our starting material. Purification procedures, as applied to pleomorphic organisms, invariably have a selective influence ; either one tends to discard the larger or the smaller elements. It is important to bear this in mind. Our own experiments were designed to determine the limiting porosity for the complete retention of typhus rickettsiae and to compare the behaviour of several different strains. It was clearly the smallest phase of the organism that would settle this limiting porosity. Hence our procedure consisted in studying the filterabilities of rickettsial suspensions, in serum broth at pH 7·8–8·0, which had already been centrifuged under known conditions to remove gross tissue particles, and, inevitably, some of the larger rickettsial bodies. We aimed at securing the most favourable conditions for filtration that the circumstances would allow.

Membranes of the " gradocol " series (Elford, 1931) have been used and the porosities are given in terms of the average pore diameter, in microns (μ) or millimicrons (mμ.). The protocols given in the following paragraphs are those of a few only of the experiments made with each strain of typhus studied. Concordant results were obtained throughout, and these have since been further corroborated in the application of the filtration method in extended typhus investigations.

Murine typhus.

Protocol 1.—Murine typhus from infected yolk sac. The several supernatants listed in Table IV were each filtered through a 0·7μ membrane. All yielded filtrates with positive rickettsial activity.

Protocol 2.—One infected yolk sac was ground up in 25 c.c. saline and the suspension divided into two portions, of which the first was spun for 15 min. at 6000 r.p.m., and the second for 15 min. at 10,000 r.p.m. The supernatants were each filtered through a 2·4μ membrane under a few cm. water pressure, and then through a 0·68μ membrane at 10 lb./sq. in. The results of tests are given in Table VI.

TABLE VI.—*Filtration of Murine Typhus from Infected Yolk Sac.*

System.	Spot counts at indicated dilutions.					
	Und.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.
Original unspun suspension	..	..	c	c	150, 200	46, 31
					200, 200	22, 36
Supernatant after 15 min. at 6000 r.m.p.	..	..	c	++	103, 128	12, 10
					137, 153	15, 17
Filterable through 0·68μ membrane	28, 10	..	..	..	..	..
	15, 22					
Supernatant after 15 min. at 10,000 r.p.m.	..	++	45, 68	2, 2	..	..
			53, 34	3, 4		
Filterable through 0·68μ membrane	101, 55	..	..	..	..	..
	54, 130					

Epidemic typhus (Breinl strain).

Protocol 1.—A suspension of infected yolk sac in serum broth was spun for 15 min. at 6000 r.p.m. The supernatant was filtered through a 2·3μ membrane and then portions of the filtrate passed through 0·75μ and 0·48μ membranes at 10 lb./sq. in. Results are summarized in Table VII.

TABLE VII.—*Filtration of Epidemic Typhus (Breinl Strain) Grown in Yolk Sac.*

System.	Spot counts at indicated dilutions.					
	Und.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.
Original suspension	..	..	..	++	150, 100 200, 100	1, 15 14, 2
Supernatant at 6000 r.p.m.	..	..	++	75, 84 80, 150	3, 4 5, 8	..
2·3μ filtrate	..	..	++	150, 200 250, 200	..	..
0·75μ ,,	..	++	69+, 500 300, d	..	..	..
0·48μ ,,	0, 0 0, 0	..	..	..	..	..

Protocol 2.—A suspension of infected mouse lung in serum broth was spun for 15 min. at 6000 r.p.m. The supernatant was filtered through a 2·3μ membrane and then 4 c.c. portions of the filtrate passed through membranes of porosities 0·75μ and 0·53μ. The results are summarized in Table VIII.

TABLE VIII.—*Filtration of Epidemic Typhus (Breinl Strain) Grown in Mouse Lung.*

System.	Spot counts at indicated dilutions.					
	Und.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.
Original suspension	..	..	..	++	38, 80 99, 49	29, 10 d, d
Supernatant at 6000 r.p.m.	..	..	++	46, 60 69, 59	6, 7 5, 6	0, 0 0, 2
2·3μ membrane filtrate	..	++	300, 300 300, 200	29, 32 37, 51	7, 7 5, 8	..
0·75μ ,, ,,	150, 200 150, 200	20, 11 29, d	5, 5 d, d	..	..	..
0·53μ ,, ,,	0, 0 0, 0	..	..	..	..	..

Epidemic typhus (Tunisian strain).

Protocol 1.—A 10 per cent. suspension of infected mouse lung in serum broth was spun for 5 min. at 2000 r.p.m. followed by ½ hr. at 7000 r.p.m. The supernatant was filtered through a 2·3μ membrane and the filtrate then passed through a 0·73μ membrane at 10 lb./sq. in. pressure. The 0·73μ filtrate was in turn filtered through a 0·5μ membrane at 10 lb./sq. in. The deposit after the 7000 r.p.m. spinning was re-suspended in serum broth and again spun for ½ hr. at 7000 r.p.m. The new deposit was ground up in a conical glass grinder and made to the original volume in serum broth. The results of tests are given in Table IX.

TABLE IX.—*Filtration of Epidemic Typhus (Tunisian Strain) from Infected Mouse Lung.*

System.	Spot counts at indicated dilutions.						
	Und.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.
Original suspension	0, 0	..	..	..	41, 17+ 31, 50	5, 7 6, 13	0, 0 0, 5
Supernatant at 7000 r.p.m.	..	..	38, 88 157, d	2, 19 12, 9	..	..	..
0·73μ filtrate	..	53, 34 23, d	4, 5 4, d	..	..	..	..
0·5μ ,,	0, 0 0, 0	0, 0 0, 0	..	..	..	..	..
Reconstituted washed deposit	..	..	..	40, 49 150, 77	3, 9 1, 6	0, 0 2, 3	..

Protocol 2.—A suspension of infected yolk sac in serum broth was spun for 15 min. at 6000 r.p.m. The supernatant was carefully withdrawn to avoid the superficial layer of fat, and then filtered through a 2·3μ membrane under a few cm. water pressure. Then portions of the 2·3μ filtrate were passed through 0·75μ and 0·5μ membranes at 10 lb./sq. in. The results are contained in Table X.

TABLE X.—*Filtration of Epidemic Typhus (Tunisian Strain) from Infected Yolk Sac.*

System.	Spot counts at indicated dilutions.						
	Und.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.
Original suspension	..	..	..	..	10, 34 23, 28	3, 3 3, d	..
Supernatant at 6000 r.p.m.	..	..	..	34, 52 56, 18	4, 7 7, 0	..	..
2·3μ filtrate	..	..	200	19, 26 28, 21	..	..	..
0·75μ ,,	..	400	72, 50 98, 30	..	..	..	..
0·5μ ,,	0, 0 0, 0	..	..	..	..	..	..

The strains of murine and epidemic typhus studied here have shown no apparent difference in general behaviour on filtration, no matter whether they have been grown in egg yolk sac or in mouse lung. A membrane of 500 mμ. porosity has proved adequate for retaining the organism completely, and this makes it improbable that the smallest phase of the rickettsiae is less than 400 mμ to 500 mμ. in diameter. (*N.B.*—The figure would correspond to the width of a rod-shaped particle.) A 700 mμ. membrane has generally yielded a positive filtrate by the fractionation procedure adopted in these studies, but there has always been a very considerable reduction in the titre of infectivity. Even when using the 2300 mμ. clarifying membrane a fraction, presumably consisting of the larger forms, of the organisms is held back. We infer from this that the organism is probably pleomorphic, or not spherical in shape, or both. Direct microscopical observations of stained smears of the infected tissue reveal that the

typhus rickettsiae are not spherical in shape, and do exhibit a degree of pleomorphism which varies at different stages of adaptation to the host tissue.

Our studies, more recently, have also included the examination of purified suspensions of typhus rickettsiae by the aid of the ultraviolet light and electron microscopes. We have had the collaboration of our colleagues Mr. J. Smiles and Mr. F. V. Welch in obtaining photographs by these methods. The estimations of size agree well with those of the filtration experiments. Thus the ultraviolet light photographs taken of suspensions in buffered saline indicated that the organism varied from 0·6 to 1·8μ in length, with a relatively uniform width of 0·45μ. On drying the organisms, and re-immersing in castor oil, the ultraviolet light photographs showed that a 25 to 33 per cent. contraction had taken place. This is a fact of great importance in interpreting the electron microscope photographs of the organisms, since these have of necessity to be made on dried preparations. Images obtained with the electron microscope confirm the order of size for the rickettsiae, but the complete analysis of our findings is beyond the scope of the present paper.

SUMMARY.

Factors influencing the viability of typhus rickettsiae contained in suspensions of murine and epidemic strains grown in egg embryos and mouse lungs have been investigated. Serum broth, of the media tested, provided conditions most favourable for the maintenance of viability, while the optimum zone of pH was from 6 to 8·5. Rickettsial suspensions, which rapidly lose activity at 0° C. and above, may be stored for months at —77° C. with little alteration in potency.

Comparative filtration experiments with the murine and epidemic typhus strains indicated a general similarity in filtration behaviour no matter whether the organism had been grown in yolk sac or mouse lung. A gradocol membrane of 500mμ. porosity completely retained all rickettsiae, while the filtrate from a 700 mμ. membrane was invariably positive following the fractionating procedure adopted. It is considered improbable that the smallest phase of the rickettsial strains here studied is less than 400 to 500 mμ. in diameter or width. The filtration data also suggest that, for each strain, the organisms are pleomorphic, which agrees with microscopical evidence.

In conclusion we would record our indebtedness to the late Miss Dora Lush for the most valued assistance rendered during the early stages of these studies.

Since this paper was submitted for publication, a paper by Anderson (1944), dealing with the survival of *Rickettsia prowazeki* in different diluents, has come to hand. Anderson finds sterile skimmed milk to be the best medium, of those he has used, for the survival of typhus rickettsiae. This confirms the earlier finding of Topping (1940).

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A BLOOD-GROUP A ANTIGEN PREPARED FROM AN A-HAPTEN OF HUMAN ORIGIN.

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THE blood group A-substance present in hog gastric mucin is without significant antigenicity when given intravenously to rabbits in doses of 0·01–1·0 mg. (Morgan and King, 1943). The A-substance, however, combines with the conjugated protein component of the O antigen of *Bact. Shigae*, and the artificial polymolecular complex thus formed possesses the power to induce the formation of potent anti-A immune-body without causing a significant increase in the animal's natural anti-B and anti-O agglutinins (Morgan, 1943). In view of the fact that the anti-A agglutinin was formed as a result of immunization with a polysaccharide complex of animal (hog) origin, it was considered of sufficient interest and importance to extend the investigation to include the production of specific anti-A sera induced by means of an artificial complex formed from an A-hapten of human origin. At that time a specific A-substance of human origin and of established purity had not been described. The examination by Morgan and van Heyningen (1944) of a number of pseudo-mucinous ovarian cyst fluids obtained from women belonging to group A who secrete in their tissue fluids the blood group substance in a water-soluble form, however, revealed that cyst fluids of this kind frequently contain a surprisingly large amount of the specific blood-group substance in a relatively pure form. The A-substance from this source was subsequently isolated (King and Morgan, 1944) and its properties compared with, and shown to be very similar to, those of the A-substance derived from hog mucin.

The present paper contains an account of the conversion of the purified non-antigenic A-substance isolated from pseudomucinous ovarian cyst fluid into a complete antigen, the immunization of rabbits with this artificial antigenic complex to yield potent anti-A sera, and the examination of these sera for their general serological characteristics, more especially for their reaction with erythrocytes of different phenotypes.

EXPERIMENTAL.

The A-substance.—The specimen of A-substance used was obtained from a single pseudo-mucinous ovarian cyst by a combination of two methods already described (Morgan and King, 1943). Full details of the method of isolation will be given elsewhere. The specimen was electrophoretically homogeneous and showed the following analytical figures: C, 44·8; H, 6·9; N, 6·0; CH_3CO, 10 per cent., and specific rotation $[\alpha]_{5461}$ + 12° (c, 0·5). The relative viscosity, η, of an 0·5 per cent. solution in 0·9 per cent. saline at 37° was 1·9 (saline, 1·00).

The "Shiga" protein component.—A part of the same preparation of conjugated protein as was used to form the antigenic complex with hog gastric mucin A-substance was employed (Morgan, 1943).

The artificial antigenic complex.—The complex was made by dissolving 50 mg. of the purified A-substance in 5 ml. of sterile saline and adding a solution (5 ml.) of the conjugated protein component (15 mg.) dissolved in 0·05 N NaOH. After standing at room temperature for 15 minutes the mixed solutions were adjusted to pH 4·5 by the cautious addition of acetic acid and made up to 26 ml. with sterile saline containing a few drops of toluene. From this slightly opalescent stock solution of the antigen, which was stored at 0–2°, suitable dilutions in sterile saline were made immediately before use.

Immunization.—Rabbits which contained natural anti-A agglutinins were used for immunization. An anti-A agglutinin titre of 32 or higher was considered suitable, provided the anti-B and anti-O titres were less than half the normal anti-A titre. The doses of the hapten and antigen preparations were given by the intravenous route at 3–4 day intervals. Bleedings were taken at 6–9 days after the last dose of antigen, and the separated serum, after heating for 20 minutes at 56°, was stored at −20° without the addition of antiseptic.

Agglutination, precipitation and haemolysis inhibition tests.—The technique used for carrying out these reactions was exactly as described earlier (Morgan, 1943).

Anaphylaxis tests.—Guinea-pigs weighing 300–450 g. were passively sensitized by the intraperitoneal injection of 3–5 ml. of anti-A rabbit serum. Two groups of guinea-pigs were used, one receiving anti-A serum (anti-A titre, 1 : 8192) induced by the artificial antigen made from cyst A-substance; the other group received the same amount of an anti-A serum of similar potency produced by means of the artificial antigen made from A-substance derived from hog gastric mucin. After an interval of 24 hours the sensitized animals received intravenously different quantities of A-substance from cyst fluid or from hog gastric mucin.

IMMUNIZATION RESULTS.

In the first instance the crude cyst substance, from which the purified A-substance was obtained, was tested for antigenicity in a group of 4 rabbits, each animal receiving six 0·5 mg. doses for the first course of immunization. A further three doses were then given and another bleeding was taken. The anti-A titres of the normal sera were 64, 128, 32 and 64. After the animals had received nine doses of the crude cyst-substance the anti-A titres had increased to 2048, 4096, 2048 and 1024 respectively. The agglutinins reactive against O-cells did not change appreciably in titre during immunization. It is evident from a consideration of these results that the crude cyst-substance possesses definite but weak antigenic power. The first preparation of purified A-substance obtained from the crude cyst-substance was likewise tested in four selected rabbits, and was found to possess even less antigenicity than the crude material. The normal anti-A agglutinin titres before and after immunization were 128, 64, 128, 128, and 512, 128, 128 and 512 respectively. The A-substance(4·9 g.) was further purified and again tested in six animals (Table I, Rabbits 670, 683, 684, 686, 689 and 701). In this experiment the normal and immune-sera were examined for anti-A, anti-B and agglutinins active against O erythrocytes. The values for each normal serum are given in Table I. The average value for the anti-A agglutinin was 90, whereas the anti-B and anti-O values were considerably lower. After the animals had received six doses, each of 0·1 mg. of the A preparation, the average anti-A titre had risen to 469, equivalent to about a five-fold increase. The average anti-B titre likewise increased and reached about ten times the normal level. The anti-O titre showed approximately a three-fold increase. Since agglutinins against all types of erythrocytes have increased during immunization with the A-substance, one must

conclude that the rise in anti-A immune-body is largely a non-specific response. The preparation of A-substance examined can be said to be without significant power to induce the formation of specific anti-A agglutinins.

After a few days rest the animals were given three doses each of 0·10 mg. of the artificial antigen made from the same specimen of A-substance that had been used above and shown to be practically devoid of specific antigenic properties. Bleedings were taken one week after the last dose of antigen; the animals were then given a further three doses of the artificial antigen and again bled one week later. Each serum sample was tested for anti-A, anti-B and agglutinins reactive against O-cells. The titration results are shown in Table I. The response of each animal was immediate and although the titre of the anti-A agglutinin obtained from animal 701 was rather low, that obtained from No. 683 reached a high value. The average anti-A agglutinin titre for all the samples collected after three doses of the antigen had been given was about 16,000, whereas the corresponding anti-B value was no more than 533 and the agglutinin titre against O-cells about 1 : 10. The anti-A titre was, therefore, about 200 times the average initial normal level and 35 times the average level found after

TABLE I.—*Showing the Result of Immunizing Rabbits with* (a) *a Blood-group A-specific Substance Obtained from a Pseudomucinous Ovarian Cyst Fluid, and* (b) *an Artificial Complex made from the A-substance.*

Rabbit number.	Titre of A, B and O agglutinins in the normal serum.			Titre of A, B and O agglutinins after giving six doses each of 0·1 mg. of A-substance.			Titre of A, B and O agglutinins after additional three doses of 0·1 mg. of artificial complex.			Titre of A, B and O agglutinins after a total of six doses of 0·1 mg. of artificial complex.		
	A.	B.	O.	A.	B.	O.	A.	B.	O.	A.	B.	O.
670	128	16	4	256	64	16	16,384	256	4	16,384	64	8
683	64	16	4	512	128	64	32,768	2048	16	65,536	2048	4
684	128	8	8	256	32	2	16,384	128	2	32,768	128	2
686	32	4	8	1024	128	32	16,384	256	32	16,384	256	4
689	128	16	8	256	128	8	8,192	128	8	32,768	256	4
701	64	8	16	512	256	16	4,096	128	16	16,384	64	16

the course of six doses of the unconjugated A-substance had been given. The average anti-B titre showed about a fourfold increase over the average value after the animals had received the A-hapten. The corresponding titre against O-cells showed little change from the normal or post hapten levels. An additional course of three doses of A-antigen produced a further rise in the specific anti-A agglutinins, the average value of 30,000 being now over 300 times as high as the initial level and about 65 times higher than the level after immunization with A-substance alone. This additional course again gave rise to no significant change in the level of anti-B agglutinins; the value of the average anti-O titre was less than 1 : 10.

From time to time further groups of rabbits have been immunized with other preparations of artificial antigen, and in every instance a powerful A-specific immune response has been obtained.

PROPERTIES OF THE ANTI-A IMMUNE-SERUM.

Action on erythrocytes of different phenotypes.

The immune-sera obtained from a group of 12 rabbits were examined by Dr. G. L. Taylor, of the Galton Laboratory, Serum Unit, Cambridge, for their action on human erythrocytes of known phenotype. The results obtained with six of these immune-sera collected after the animals had received three doses each of 0·05 mg. of the antigen are set out in Table II. The results are similar to those given by the anti-serum to

an artificial antigen made from hog gastric mucin A-substance (Morgan, 1943). The titration values show that the immune-body contains a common *alpha*-agglutinin component which reacts not only with A_1 and A_1B erythrocytes, but also with cells of the phenotype A_2 and A_2B. It is to be noted, however, that in most instances there is a greater difference in the A_1 and A_2 titres than was found for the immune-sera produced against the artificial antigen made from the A-substance isolated from hog gastric-mucin.

TABLE II.—*Showing the Agglutination Titres of Rabbit Immune-sera Tested against Human Erythrocytes of Different Phenotypes.*

	Titre of serum against human erythrocytes. Rabbit number.					
Group of test-cells.	649.	676.	680.	698.	710.	712.
A_1	32,768	131,072	65,532	131,072	131,072	262,144
A_2	4,096	4,096	8,192	65,536	8,192	32,768
A_1B	8,192	8,192	8,192	32,768	8,192	32,768
A_2B	2,048	8,192	4,096	16,384	4,096	16,384
B	128	256	128	64	64	128
O	4	8	2	2	2	2

The large difference in the agglutination titre against A_1 and A_2 erythrocytes suggested that a reagent for the differentiation of A_1 cells from those of sub-group A_2 could be prepared either by (1) absorption of the common *alpha*-agglutinin with A_2 erythrocytes, until the agglutinin reactive against cells belonging to this sub-group were completely eliminated, or (2) by simple dilution of the immune-serum until the diluted serum failed to react with A_2 cells, but gave strong agglutination with A_1 cells. Several reagents reactive against A_1 cells only and of useful potency (A_1 titre 1 : 128) were prepared by method (1). By means of the method (2), several effective antibody preparations were also obtained. For example, immune-serum from Rabbit 676 (Table II) was filled, in 0·2 ml. quantities, into ampoules, was frozen at −15°, and evaporated to dryness *in vacuo* from the frozen state. The dried serum was dissolved in saline when required and, after suitable dilution (1 : 5000 with serum 676), was ready for immediate use for the differentiation of A_1 and A_2 cells; the former cells were strongly agglutinated, the latter remained unagglutinated. A more detailed examination of anti-sera reactive against A_1 cells and without action on A_2 erythrocytes is being undertaken.

Influence of A-substance on inhibition of agglutination.

The influence of the purified cyst A-substance, hog gastric mucin A-substance and of partially purified A-substance isolated from human saliva on the agglutinating action of the immune-serum engendered by the artificial antigen made from cyst A-substance was investigated. The test-serum used showed a titre of 192 against

TABLE III.—*The Agglutination of A_1-erythrocytes by an Immune-serum Prepared against Ovarian Cyst A-substance after Treatment of the Serum with A-substances from Different Sources.*

A-substance derived from—	Dilution (×10⁴) of A-substance causing inhibition of agglutination.									
	1 : 1.	1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.	1 : 64.	1 : 128	1 : 256.	Saline control.
Ovarian cyst	0	0	0	0	0	0	1	2	3	4
Hog gastric mucin	0	0	0	0	0	1	2	3	4	4
Human saliva	0	0	1	2	3	3	4	–	–	4

Degrees of agglutination: 0, no agglutination; 1, a few groups of 2–3 cells; 2, larger groups with many free cells; 3, small clumps visible to the naked eye; 4, massive agglutination.

A-cells, and was therefore of similar strength to the "standard" anti-A agglutinin (human) reagent employed throughout the earlier work (Morgan, 1943; Morgan and King, 1943; Morgan and van Heyningen, 1944). The results are given in Table III, and show that the power of each of the purified A-substances to inhibit the agglutination of A cells by the serum is of the same order, and, as would be expected, is considerably greater than that shown for the impure A-substance derived from saliva.

The haemolytic action of the immune-serum.

The normal and immune-sera were also examined for immune-body that would lyse sheep red-cells in the presence of complement. The average haemolytic titre of the normal serum samples of the six rabbits given in Table I was 120; the average value after the animals had received six doses of the A-substance decreased to 100, but after receiving the first course of three doses of the artificial antigenic complex, the average haemolytic titre increased to 1230. There was no significant rise above this figure after a further three doses of the antigen had been given. The haemolytic properties of a pooled specimen of the immune-sera were compared with those of four other haemolytic sera by means of the haemolysis inhibition test. In each instance 2 M.H.D. of serum were employed as test reagent, and in order to obtain further information, a specimen of hog gastric mucin A-substance was included with the cyst A-substance as an inhibiting agent. The results of these experiments are shown in Table IV. It will be seen that the haemolytic action of the cyst A-substance immune-serum was qualitatively and quantitatively identical with the haemolytic action of the hog mucin A-substance immune-serum, and with the anti-A serum induced by human A-erythrocytes. As little as 0·125 μg. of each of the A-substances caused almost complete inhibition of lysis of sheep cells by 2 M.H.D. of the three A-specific immune-sera tested, whereas the A-substances are shown to be devoid of power to inhibit the lytic action of an anti-guinea-pig kidney (Forssman) or an anti-sheep red cell serum.

TABLE IV.—*Showing the Inhibition of Haemolysis of Sheep Cells in Different Types of Immune-sera (2 M.H.D.) by A-substance Isolated from* (a) *Pseudomucinous Ovarian Cyst and* (b) *Hog Gastric Mucin.*

Rabbit serum prepared against—	Dilution ($\times 10^6$) of ovarian cyst A-substance causing inhibition of haemolysis.						Saline control.	Dilution ($\times 10^6$) of hog mucin A-substance causing inhibition of haemolysis.						Saline control.
	1:1.	1:2.	1:4.	1:8.	1:16.	1:32.		1:1.	1:2.	1:4.	1:8.	1:16.	1:32.	
Cyst A-substance artificial antigen	0	0	1	3	4	4	4	0	0	0	2	4	4	4
Hog mucin A-substance artificial antigen	0	0	1	4	4	4	4	0	0	1	3	4	4	4
Human erythrocytes	0	0	1	3	4	4	4	0	0	0	1	3	4	4
Guinea-pig kidney	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Sheep red cells	4	4	4	4	4	4	4	4	4	4	4	4	4	4

0, No haemolysis; 1, trace of haemolysis; 2, definite haemolysis; 3, almost complete haemolysis; 4, complete haemolysis.

Precipitation of the immune-serum with A-substance.

The undiluted cyst A-substance immune-serum gives distinct precipitation with the homologous A-substance at a dilution of 1 : 100,000, less definite precipitation with the A-substance isolated from hog gastric mucin and with the active saliva substance obtained from secretors belonging to group A. No reaction is given with the active blood-group substances occurring in the saliva of secretors belonging to groups B or O. B-substance isolated from active pseudo-mucinous ovarian cyst fluids and

saliva substance from non-secretors of groups A, B and O gave no precipitation at a dilution of 1 : 1000, 1 : 10,000 and 1 : 100,000 with the immune-serum produced against the artificial antigen made from cyst A-substance. A quantitative determination of the amount of anti-A immune-body present was carried out, according to the method of Heidelberger, Kendall and Soo Hoo (1933), on the immune-sera obtained from Rabbits 698, 710 and 712. Duplicate 3 ml. amounts of the serum were treated with 0·05 ml. portions of 1 : 2000 solution of the A-substance derived from ovarian cyst fluids. After each addition of the A-substance the solutions were thoroughly mixed, incubated at 37° for 1 hour and kept at 0° for several hours. The solutions were centrifuged at 0° to remove the insoluble A-substance-antibody complex, and again tested for the presence of antibody by the addition of further quantity of the A-substance, incubation and cooling to 0°. The first addition of the A-substance that failed to give rise to a further quantity of hapten-antibody complex was taken as the end-point, and it was found that at this stage the supernatant solutions possessed no detectable anti-A agglutinin content as measured by their agglutinating action on A_1 cells. The insoluble A-hapten-antibody precipitate was thoroughly washed with ice-cold saline, dissolved in dilute NaOH and the total nitrogen estimated. Sera 698, 710 and 712 contained 0·89 mg., 0·51 mg., and 1·05 mg. of antibody protein per ml. respectively. These figures are corrected for the nitrogen content of the A-substance in the A-hapten-antibody complex. A parallel experiment was carried out in which A-substance derived from gastric mucin was used in place of the cyst A-substance. The amount of insoluble A-hapten-antibody complex formed, however, was in each instance less than when cyst A-substance was used. The determination of the anti-A immune-body by means of the gastric mucin A-substance indicated that 0·21 mg., 0·32 mg. and 0·58 mg. of antibody protein was present per ml. of sera 698, 710 and 712. The end-point of the precipitation reaction was less definite when gastric mucin A-substance was employed than when cyst A-substance was used as precipitating reagent. Furthermore, it was found that after the gastric mucin A-substance-immune-body complex had been completely removed from the immune-serum, the addition of A-substance derived from ovarian cyst fluid failed to cause a further precipitate to occur, although the addition of cyst A-substance to the serum in the first instance produced a considerably larger precipitation of antibody than was brought about by the addition of A-substance obtained from hog gastric mucin.

Anaphylaxis.

It was shown (Morgan, 1943) that guinea-pigs passively immunized with an anti-A rabbit serum induced by immunization with an artificial A-antigen of animal (hog) origin failed to develop anaphylactic shock when given intravenously the homologous A-hapten. No satisfactory explanation was forthcoming to account for the absence of anaphylaxis, and since that time the experiment has been repeated, using a larger sensitizing dose of immune-serum. With relatively large doses (10 mg.) of the gastric mucin A-substance all of the animals sensitized developed anaphylaxis, and two of the guinea-pigs died of anaphylactic shock within three minutes of receiving the A-substance intravenously. Two guinea-pigs that received 5 mg. of the A-substance, however, showed no symptoms of anaphylaxis. Similar experiments have now been carried out using rabbit immune-serum engendered by the artificial antigen prepared from cyst A-substance. Each of a group of 6 guinea-pigs was given 3 ml. of a potent anti-A serum (A titre, 1 : 8192) intraperitoneally, and 24 hours later the animals received 5 mg. of the cyst A-substance intravenously ; five of the animals died of typical anaphylactic shock within three minutes of receiving the A-substance. Two other animals, after passive sensitization, received a dose of 1 mg. only of the A-substance ;

one died of anaphylactic shock, the other showed slight but definite symptoms of anaphylaxis. Another group of 4 guinea-pigs was sensitized with the same serum, and each animal was given intravenously 5 mg. of the A-substance isolated from hog gastric mucin. Two animals died within four minutes of typical anaphylactic shock; the remaining two guinea-pigs developed definite symptoms of anaphylaxis, but did not die. A further group of five guinea-pigs was likewise passively immunized with a gastric mucin A-substance immune-serum (A titre, 1 : 8192), and the animals were given intravenously 5 mg. of cyst A-substance; one guinea-pig died in four minutes, the remaining animals showed characteristic symptoms of anaphylaxis. The results of these experiments indicate that anaphylaxis is more readily induced by cyst A-substance in animals sensitized with an anti-A serum engendered against the cyst A-substance artificial antigen, than in animals sensitized by an anti-A serum produced by the artificial antigen made from gastric mucin A-substance and shocked with an intravenous dose of the homologous gastric mucin A-substance.

DISCUSSION.

As a result of the work described in this paper it can be stated that the A-substance in pseudomucinous ovarian cyst fluids combines with the conjugated protein component of the O antigen of *Bact. shigae* to form a complex which, when given intravenously to rabbits, induces the formation of A-specific immune-body. In this respect, therefore, A-substance of human origin behaves like the A-substance isolated from hog gastric mucin and pepsin. A detailed examination of twelve typical anti-A immune-sera engendered by the cyst A-antigen has shown that they agglutinate A_1 erythrocytes at very high dilutions, in certain instances at dilutions as high as 1 : 250,000. The titre against A_1B cells on the other hand is less, and is usually found to be between 8000 and 32,000. Similar but slightly lower values are found for the agglutinin titres against A_2 and A_2B cells. The results are similar to those given by immune-sera produced against an artificial antigen prepared from hog gastric mucin (Morgan, 1943), and show that the immune-body contains a common *alpha*-component which reacts strongly with A_2 and A_2B erythrocytes as well as with cells of the phenotype A_1 and A_1B. It is to be noted, however, that there is a greater difference between the values of the A_1 and A_2 titres in those anti-A sera induced by cyst A-antigen than in those produced against the artificial antigen prepared from hog gastric mucin A-substance. Indeed, a reagent for the differentiation of A_1 and A_2 cells has been prepared from certain of the immune-sera induced by means of the artificial antigen made from cyst A-substance. The agglutination results obtained with these absorbed sera suggest that a considerable part of the immune-body engendered by the antigen formed from cyst A-substance is $alpha_1$ in character, and it has been observed that removal of the common *alpha*-agglutinin by means of A_2 cells leads to the preparation of quite potent anti-A immune-body reactive against A_1 cells only. In our experience the majority of anti-A rabbit sera produced as a result of immunization with three or six intravenous doses, each of 0·05 mg. of an artificial antigen made from A-substance derived from hog gastric mucin or pepsin, do not possess a useful titre of an agglutinin reactive against A_1 cells after the sera have been absorbed with A_2 cells. The difference in the nature of the response with the two types of antigen may be related to a difference in the make-up of the A-substance from the two sources. That serological differences probably exist in the A-substances of different origin is suggested from the observations of Morgan and van Heyningen (1944), who showed that whereas the carefully purified A-substance from hog gastric mucin inhibits the action of natural anti-O cattle agglutinin on O cells, A-substance from the majority of pseudomucinous cysts has no such action.

A further difference between A-substance derived from hog gastric mucin and that obtained from ovarian cyst fluid becomes evident when the amounts of A-immune-body precipitable by these substances from an immune-serum produced by an A-antigen made from cyst A-substance are compared. The quantitative examination of three anti-A-immune sera produced against cyst A-substance antigen, and exhausted of agglutinins by means of absorption with the unconjugated A-substances, showed that the homologous cyst A-substance precipitated from solution considerably more agglutinin than did the hog gastric mucin A-substance.

Anaphylaxis can be induced in guinea-pigs that have received an appropriate intraperitoneal dose of anti-A serum produced against cyst A-antigen, by giving either an intravenous dose of hog mucin A-substance or of cyst A-substance. Guinea-pigs that have been sensitized with an immune-serum prepared against an antigen derived from hog-mucin A-substance likewise show the immediate onset of anaphylaxis when given intravenously cyst A-substance or the homologous gastric mucin A-substance.

In considering differences which might exist between the A-substance of ovarian cyst fluids and of hog gastric mucin, it must not be forgotten that the cyst A-substance probably represents the naturally occurring water-soluble A-substance essentially unchanged, whereas the hog mucin A-substance used throughout the work was derived from commercial materials, the method of preparation of which is uncertain and could, therefore, have given rise to significant changes in the properties of the original native A-substance. Thus changes brought about by the method of preparation might be responsible for certain of the differences observed.

SUMMARY.

(1) The formation of an antigenic complex by the combination of the non-antigenic A-substance isolated from pseudomucinous ovarian cyst fluid with the conjugated protein component of the specific O somatic antigen of *Bact. Shigae* is described.

(2) The artificial antigen induces in the rabbit the formation of extremely potent immune-body, which agglutinates human erythrocytes belonging to group A, but not those belonging to groups B or O.

(3) An agglutinin reactive against A_1 erythrocytes but not against A_2 cells can be obtained from certain of the potent anti-A immune-sera.

(4) The anti-A sera cause the haemolysis of sheep cells in the presence of complement. The haemolysis is inhibited by the homologous A-hapten, by A-substance isolated from hog gastric mucin and by the A-substance in the saliva of persons secreting this factor.

(5) Some observations which suggest differences in the A-substances obtained from pseudomucinous ovarian cyst fluids and from hog gastric mucin are discussed.

Our thanks are due to Dr. G. L. Taylor, of the Galton Serum Unit, Cambridge, for the titration results shown in Table II.

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THE EFFECT OF PENICILLIN ON *TREPONEMA PALLIDUM* INFECTION IN RABBITS.*

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THE chemotherapeutic value of penicillin in certain bacterial infections is now well established. Its main advantage is its very low toxicity, so that high bacteriostatic concentrations can be borne in the blood stream and tissues without endangering life. The use of penicillin in the treatment of syphilis would therefore be a distinct advance on current methods of treatment, which have the great drawback of having undesirable side effects. That penicillin has a curative action on human syphilis has already been reported by Mahoney, Arnold and Harris (1943), who treated four early cases by 4-hourly intramuscular injections, giving a total of 1,200,000 units of penicillin in 8 days; spirochaetes disappeared from the primary lesion within 16 hours, and the serological tests became negative within a few weeks. These authors also report that they are proceeding with an investigation on the penicillin therapy of rabbit syphilis, and state that penicillin has some spirochaeticidal power, but that there are frequent failures in short courses of treatment which utilize minimal amounts of the drug. Although no details of these experiments are given, it would appear that there was some irregularity in the response of the rabbit lesions to penicillin.

In the experiments to be reported here we have found great variation in the curative action of penicillin on experimental syphilis in the rabbit, with the result that we have so far been unable to define the conditions necessary for complete cure. We have, however, determined the dose of penicillin required to cause the disappearance of spirochaetes from rabbit lesions within 24 hours, and have adduced some evidence to account for the difficulty in curing *Treponema pallidum* infection in rabbits with short courses of penicillin therapy.

MATERIAL AND METHODS.

The strain of *Treponema pallidum* used was first passaged from a human case of syphilis by Truffi (1909), and was used by Ehrlich in his classical work on salvarsan. It was obtained from Levaditi in 1931, and has been maintained since then in rabbits in the Bland-Sutton Institute of Pathology. Passage is effected by inoculating both testicles with grafts of testicular chancre by means of a hollow needle and trocar 2 mm. in diameter. The lesions become palpable 2 to 3 weeks after inoculation, forming firm tumour-like nodules, which increase in size slowly but progressively. They usually ulcerate after a further 5 to 10 weeks but they occasionally regress, and this must be borne in mind in assessing the value of treatment.

* This work was done on behalf of the Penicillin Committee of the Middlesex Hospital under the auspices of the Medical Research Council.

The lesions selected for treatment had all attained a long diameter of 1·0 to 2·0 cm., and samples aspirated into a few drops of saline and examined by dark-ground illumination all showed 5 to 100 active spirochaetes per field. The effect of treatment was followed by measurement of the lesions and by dark-ground examinations at 6 and 24 hours after the first injection, daily for 5 days, and then at longer intervals. The penicillin was dissolved in glass-distilled water in a concentration of 1000 or 2000 units per c.c., and the injections were given intramuscularly. In each experiment the potency of the penicillin solution was checked, and in a number of rabbits the penicillin content of the blood serum was estimated by titration against the Oxford staphylococcus.

RESULTS.

The details and results of the therapeutic experiments are shown in Table I. In all but one of the 9 treated rabbits there were lesions in both testicles, so that the effect of treatment was observed in 17 lesions. Rabbits 1 to 6 received treatment on one day only, and are arranged in the order of increasing doses of penicillin. In Rabbits 2 to 6 the penicillin dose was divided into 3 equal doses administered at intervals of 3 hours in order to ensure a more prolonged action of the drug. Rabbits 7, 8 and 9 received daily treatment for 4 or 5 days.

Disappearance of spirochaetes.

A diminution in the number of spirochaetes in dark-ground preparations was already apparent at 6 hours after the commencement of treatment with the larger doses of penicillin used in Rabbits 4, 5 and 6, but in no instance was there at this stage the complete absence of active spirochaetes that can be obtained with an arsenical drug (McIntosh and Fildes, 1911). At 24 hours all the treated lesions showed either a disappearance of active spirochaetes or a reduction in their numbers. As is shown in Table I, active spirochaetes disappeared from 15 of the 17 lesions at intervals up to

TABLE I.—*Summary of Experiments.*

Rabbit No.	Duration of infection in days.	Penicillin treatment. Individual dose in units per kg.	Number of doses.	Total dose in units per kg.	Lesions.	Effect on spirochaetes: D. = disappeared. R. = reappeared.	Effect on lesion.
1	88	400	1	400	1	D. 4 days	Complete regression.
2	34	500	3 3-hourly	1500	1	D. 5 days	„ „
					2	D. „	„ „
3	53	1000	„	3000	1	D. 24 hrs.	„ „
					2	„	„ „
4	50	1500	„	4500	1	„ R. 42 days	Temporary regression.
					2	„ „	„ „
5	26	2500	„	7500	1	„ „	„ „
					2	„ „	„ „
6	57	10,000	„	30,000	1	„ R. 49 days	„ „
					2	„	Complete regression.
7	32	500	5 daily	2500	1	Dimished for 10 days	No effect.
					2	„ „ 5 „	„
8	45	1000	3 3-hourly on each of 4 successive days	12,000	1	D. 7 days. R. 35 days	Temporary regression.
					2	„ 5 „ „	„ „
9	45	1000	Ditto	12,000	1	„ 5 „ „	„ „
					2	„ 7 „ „	„ „

7 days. In the remaining two lesions, in Rabbit 7, which received only 500 units per kg. of penicillin daily for 5 days, the spirochaetes diminished temporarily, being reduced from 5 and 100 per dark-ground field to only 1 on the fourth day of treatment. A complete disappearance of active spirochaetes within 24 hours was obtained in 8 lesions in Rabbits 3 to 6, all of which received a total dose of at least 3000 units per kg. However, the results in Rabbits 8 and 9 show that a dose of 3000 units per kg. may be insufficient to cause an early disappearance of spirochaetes even when repeated on successive days.

It may be concluded from these experiments that a dose of penicillin as low as 500 units per kg. may cause the eventual disappearance of spirochaetes from rabbit lesions, and that the minimum dose necessary to cause the disappearance of active spirochaetes within 24 hours is 4500 units per kg. given in 3 equally divided doses at intervals of 3 hours. It is to be noted, however, that spirochaetes had reappeared in 9 out of 15 lesions when they were examined 30 to 48 days after they had first disappeared.

Curative action.

There was no interruption of the growth of the two lesions of Rabbit 7, in which spirochaetes were constantly present. All 15 lesions, in which the spirochaetes disappeared, became softer and smaller, their long diameters being reduced by 1/4 or 1/3 by the 7th day of treatment. In 6 lesions this process continued until they were no longer palpable at 30 to 40 days and further examinations showed no recrudescence. A similar rate of regression was observed in the remaining 9 lesions for the first 2 or 3 weeks, but they subsequently became firmer and resumed their growth. In all these recurrent lesions spirochaetes were found in large numbers at the times indicated in Table I.

The most striking feature of these results is the lack of relationship between the fate of the lesion and the dose of penicillin or the time at which spirochaetes disappeared. Thus, doses of 400 to 3000 units per kg. cured 5 lesions in Rabbits 1, 2 and 3, whereas treatment for 4 days at a daily dose level of 3000 units per kg. failed in Rabbits 8 and 9. There were also failures in 5 out of 6 lesions in Rabbits 4, 5 and 6 with larger doses which caused a prompt disappearance of spirochaetes. This variability in response to treatment may be largely due to some of the rabbits being more resistant to the infection, as would appear to be the case in Rabbit 1, where the lesion was of moderate size and unulcerated when it was treated 88 days after inoculation. On the other hand, the result in Rabbit 6, where one lesion was cured and the other relapsed after a total dose of 30,000 units per kg., would appear to indicate that success or failure may also depend to some extent on local conditions in the lesion itself.

Penicillin blood levels.

The penicillin content of the blood serum of 5 treated rabbits at intervals after the intramuscular injection of graded doses is shown in Fig. 1, where penicillin content is expressed by the 2-fold serial dilution required to inhibit completely the growth of the Oxford staphylococcus. It will be noted that the bacteriostatic levels are roughly commensurate with the doses given with the exception of those in the Rabbit 5, which are relatively high. These bacteriostatic levels, however, compare unfavourably with those obtained in man, where similar levels can be produced with much smaller doses. Using the same technique we have usually obtained bacteriostatic titres of 1/16 at 15 minutes and 1/1 at $2\frac{3}{4}$ hours after the intramuscular injection of 20,000 units in adult patients, a dose equivalent to about 300 units per kg. On the other hand,

as can be seen in Fig. 1, a dose of 1500 units per kg., or 5 times more penicillin has been required to produce similar bacteriostatic titres in the blood serum of the rabbit.

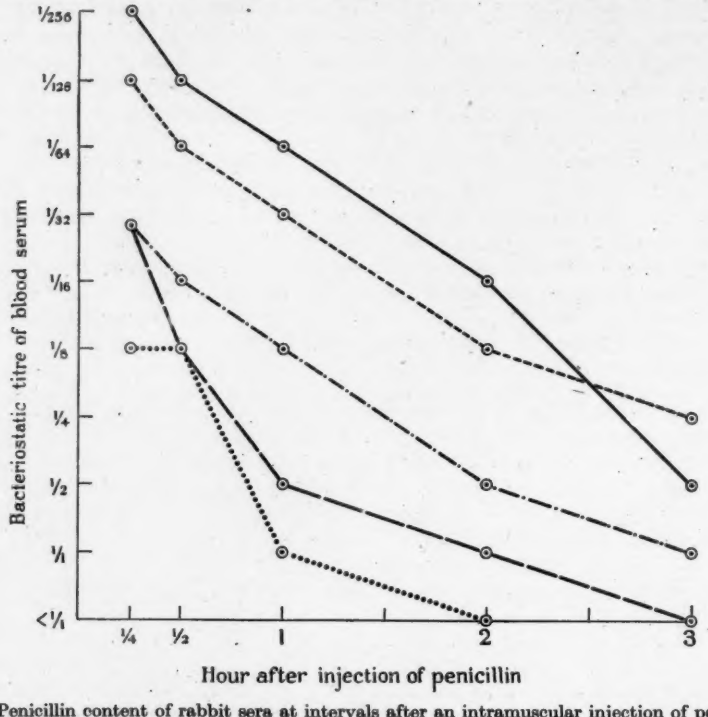

FIG. 1.—Penicillin content of rabbit sera at intervals after an intramuscular injection of penicillin. Penicillin content is expressed as the highest 2-fold dilution that completely inhibits the growth of the Oxford staphylococcus in a peptone broth medium.

Rabbit 6, 10,000 units per kg.
Rabbit 5, 2500 units per kg.
Rabbit 4, 1500 units per kg.
Rabbit 3, 1000 units per kg.
Rabbit 2, 500 units per kg.

DISCUSSION.

An evaluation of the results of these experiments can be most readily made by comparison with therapeutic tests of arsenical drugs, of which a large number have been carried out in these laboratories. In these tests it has been customary to follow the practice of McIntosh and Fildes (1911), and to regard the minimum dose required to bring about the disappearance of active spirochaetes in 24 hours as the minimal effective dose. This dose of an arsenical drug has borne a close relation to the minimum curative dose, since it entirely cures 70 per cent. of lesions, and whole multiples of this dose have been invariably curative. With neoarsphenamine and mapharside the minimal effective dose in rabbits lies between 6 and 9 mg. per kg., which is equivalent to a dose of 0·4 to 0·6 g. in adult man, so that it corresponds closely to the single doses currently used in the treatment of human syphilis.

On the basis of the experiments with arsenical drugs the minimal effective dose of

penicillin in rabbits, as determined here, is 4500 units per kg., given in 3 equal 3-hourly doses, which would be equivalent to 300,000 units in adult man. However, it has been shown here that five times more penicillin is required to produce blood levels in rabbits comparable to those obtained in man, so that the equivalent effective dose in man would be 60,000 units or 3 3-hourly doses of 20,000 units. An 8-day course at this dosage rate would give a total of 1,280,000 units, which corresponds very closely to that found successful in the treatment of human syphilis by Mahoney, Arnold and Harris (1943). It would therefore appear that, as with the arsenical drugs, the minimal effective dose of penicillin in the rabbit, as defined above, would appear to give a measure of the dosage required in the treatment of human syphilis. Regarding the curative action of penicillin on experimental syphilis, it has been shown that relatively small doses cured lesions in certain rabbits, whereas only 3 out of 8 lesions were cured in rabbits receiving doses sufficient to bring about a prompt disappearance of spirochaetes.

These results would therefore indicate that in rabbits a short course of 3 3-hourly doses of penicillin at an appropriate dosage level has an immediate effect comparable to a single appropriate dose of an arsenical drug, but that a relatively much higher dosage level of penicillin would be required to produce a permanent effect. This would appear to be due to the fact that penicillin is rapidly excreted, whereas arsenical drugs tend to be retained in the tissue of the host. The wide divergence between the doses of penicillin required to produce immediate and permanent effects has also been found to hold in *Spirillum minus* infection in mice, where 10 units are sufficient to clear the spirilla from the blood in 24 hours, whereas a dose of 1000 units is required to effect cure (Lourie and Collier, 1943).

The experimental evidence would therefore appear to indicate that large doses of penicillin would be necessary to cure syphilis in man even when allowance is made for the more rapid excretion of injected penicillin in the rabbit. However, the results of Mahoney, Arnold and Harris (1943) show that penicillin dosage comparable to that used in the treatment of bacterial infections is efficacious in early human syphilis. This apparent discrepancy is in all probability due to differences in the type of lesion, which in the rabbit is relatively large and of a dense cartilaginous consistency, and therefore not readily penetrated by drugs from the blood stream. There still remains the possibility, however, that relapses may occur in human cases treated with apparently adequate doses of penicillin, but this danger will be minimized by serological control. Even if such relapses are of frequent occurrence, penicillin will have its place as a valuable adjuvant to other methods of treatment.

SUMMARY.

Rabbits infected with *Treponema pallidum* were treated with intramuscular injections of penicillin in doses ranging from 400 to 30,000 units per kg.

The dose required to cause a disappearance of active spirochaetes from dark-ground preparations in 24 hours was 4500 units per kg.

A curative effect was obtained in certain rabbits, but there were relapses in other rabbits receiving doses of 4500 to 30,000 units per kg.

In the rabbit, 5 times as much penicillin per kg. was required to produce bacteriostatic titres in the blood serum comparable to those produced in adult man by intramuscular injection.

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THE THYMOL TURBIDITY TEST AS AN INDICATOR OF LIVER DYSFUNCTION.

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THIS test, which has been the subject of a preliminary report elsewhere (Maclagan, 1944*c*), arose in the course of work on the serum colloidal gold reaction (Maclagan, 1944*a*, *b*). In an attempt to inhibit the growth of moulds in the barbitone buffer (pH 7·8) a crystal of thymol was at first added to the solution, and as the thymol gradually dissolved it was noted that the buffer acquired the property of producing a turbidity or precipitate with certain sera. These sera were all from patients with parenchymatous liver disease and also gave positive gold reactions, and it soon became evident that there was a close correlation between the thymol turbidity and the gold test. The maximum turbidity was produced by saturating the buffer with thymol with the aid of heat (see under "Method"), and further experiments were conducted with these thymolized solutions.

EXPERIMENTAL.

pH and ionic strength.

TABLE I.—*Turbidity in Arbitrary Units Produced by Adding 3 ml. of Thymolized Buffer to 0·05 ml. of Serum under Different Conditions.*

	pH. 4.	5.	6.	7.	8.	9.	Ionic strength.
Normal	3	18	12	4	1	0	0·02
Infective hepatitis	3	21	21	14	12	3	0·02
Normal	2	8	6	2	0	0	0·05
Infective hepatitis	2	10	10	7	6	0	0·05
Normal	1	7	5	1	0	0	0·10
Infective hepatitis	1	7	6	4	2	0	0·10

It will be seen from Table I that variations in the pH and the ionic strength of the medium have as striking an effect upon the thymol turbidity as they have on the gold test. Raising either of them diminishes the turbidity within the limits shown. With most sera turbidity is maximal between pH 5 and 6, but as with the gold reaction, the difference between normal and pathological (infective hepatitis) sera is best demonstrated at low ionic strength in the neighbourhood of pH 8.

Results with related substances.

A number of other compounds were tested for their ability to produce a difference in turbidity when added to normal and to hepatitis serum at pH 7·8. They have been listed under three heads:

Positive.—Phenol, cresols, xylenol, naphthols, carvacrol, resorcinol, naphthoresorcinol, hydroquinone.

Weakly positive.—8-oxy quinoline, nitro-phenols, naphthylamine, tyrosine, desoxycholic acid, menthol, ethyl alcohol (50 per cent.) butyl alcohol (saturated).

Negative.—Disodium phenyl phosphate, salicylic acid, benzoic acid, phthallic acid, hippuric acid, picric acid, p-toluene sulphonic acid, trichloracetic acid, tungstic acid.

It appears from these lists that the effect is shown principally by phenol and its substitution products, although a few other compounds gave weaker positive results. Some groupings evidently interfere, e.g. the carboxyl group in salicylic acid, and the hydroxyl group must be free (phenyl phosphate neg.). The concentrations of the different compounds required varied widely, but there was a definite relationship between the molecular weight, the solubility, and the precipitating power.

TABLE II.—*Concentration of Substituted Phenols Required to Produce Equal Turbidities with Hepatitis Serum at pH* 7·8, $\mu = 0{\cdot}01$.

These solutions gave no appreciable turbidity with normal serum.

Compound.	Concentration (gm.%).	Solubility (gm.%).	Molecular weight.	Ratio $\frac{\text{solubility}}{\text{concentration}}$
Phenol	1·5	5·1	94	3·4
Cresols (o, m, and p)	0·75	1·8–2·5	108	2·8
Xylenol (1, 2, 4)	0·40	0·79	122	2·0
Naphthol (α)	0·10	0·11	144	1·1
Thymol	0·11	0·11	150	1·0
Carvacrol	0·12	0·12	150	1·0

It is evident from Table II that in this series with increasing molecular weight the solubility falls off more rapidly than the concentration required for effective precipitation, so that their ratio falls from 3·4 for phenol to 1·0 for thymol. Higher homologues such as cholesterol are thus too insoluble to give a positive result. These considerations at once recall the Pandy test for globulin in cerebrospinal fluid, which employs saturated phenol, and the cephalin-cholesterol flocculation test of liver function (Hanger, 1939). It seems extremely probable that both these tests depend upon the same principle, the function of the cephalin being to keep the cholesterol in solution. On this basis thymol occupies a convenient intermediate position for use with serum, as it is just soluble enough to produce a satisfactory result in saturated solution. Being effective in low concentration, it has the additional merit of simplifying the analysis of the precipitate.

Further trials on a number of typical sera showed that none of the other compounds mentioned was superior to thymol in distinguishing normal from hepatitis serum, and thymol had a definite advantage over phenol and the cresols, as it was less liable to give turbidities with sera from cases of obstructive jaundice. After many experiments involving slight alterations in the pH and the ionic strength of the medium the following technique was finally adopted.

METHOD.

Thymol buffer, pH 7·8, $\mu = 0{\cdot}01$.

Add 500 ml. of distilled water to 1·38 g. of barbitone, 1·03 g. of sodium barbitone, and approximately 3 g. of thymol. Heat just to boiling point, shake well, and cool thoroughly. The mixture should now be turbid. Seed with a small amount of powdered thymol crystals, shake, and allow to stand overnight at a temperature of 20 to 25° C. Finally, again shake well (to avoid supersaturation), and filter the clear solution from the crystalline deposit. It is not possible to shorten this technique by weighing out an exact amount of thymol, because there is some interaction between the thymol and the barbitone on heating, presumably esterification, and an excess of thymol must be present to ensure saturation. The solubility of thymol is significantly

reduced below 20° C. ; the buffer should therefore be stored in a warm place in cold weather.

Procedure.

Measure one volume of serum (0·05 ml. from a 0·2 ml. graduated pipette with a waxed tip) into a dry tube and add 60 volumes of buffer (3 ml.). Allow to stand for half to one hour, and compare in a comparator with a black line on a white background against the turbidity standards of Kingsbury *et al.* (1926). When the result is positive flocculation frequently occurs on standing overnight, but this is not an essential part of the test. If the turbidity exceeds the 100 mg. per cent. standard, dilute with a further measured volume of buffer as required. The result is expressed in arbitrary units equal to the appropriate standard divided by ten with allowance for dilution. The standard dilution is 1 to 60, so that if the final dilution is 1 to 120 and the mixture then matches the 70 mg. per cent. tube, the result is 14 units. Expressed as a formula :

$$\text{Units} = \frac{\text{Standard tube reading} \times \text{Final dilution of serum}}{600}.$$

Normal limits are 0 to 4 units. The reaction is partly abolished by heating to 56° C. for 30 minutes, so that inactivated sera cannot be used. The turbidity standards referred to are those in common use for urine protein estimation, and can be purchased from Messrs. Gallenkamp (King and Haselwood, 1936). They should be checked every few months against diluted serum of known protein content, using one volume of diluted serum to 3 volumes of 3 per cent. sulphosalicylic acid.

Composition of the Precipitate.

In five typical positive cases 2 ml. of serum were treated with 100 ml. of buffer and allowed to stand overnight. The clear supernatant fluid was removed by suction and the precipitate was centrifuged into a weighed tube, using the angle centrifuge at 3000 revs. per minute for one hour. The precipitate was washed with buffer and re-centrifuged, and after decanting the supernatant fluid the tube was weighed wet, dried to constant weight over P_2O_5 at room temperature (about 4 days) and re-weighed. The wet weight gave the volume of buffer included, and permitted a correction for its nitrogen and thymol content. The dried precipitate was finally dissolved in 5 ml. of 0·1 N NaOH, and aliquots were taken for determination of nitrogen (Peters and Van Slyke, 1932, **2**, 353), phosphorus (p. 882), thymol (p. 657), and cholesterol (p. 504). The corrected results are shown in Table III.

TABLE III.—*Composition of Precipitate in Five Cases.*

Diagnosis.	Infective hepatitis.	Infective hepatitis.	Infective hepatitis.	Rheumatoid arthritis.	Cirrhosis.	Averages.
Mg. of ppt. per 100 ml. serum	1050	1400	1300	1700	1330	..
Units of turbidity	8	16	9	27	13	..
Mg. % per unit	130	88	145	63	102	106
N %	5·4	4·7	6·7	6·4	7·2	6·0
Protein %	33·8	29·4	41·8	40·0	45·0	37·5
P %	0·45	0·47	0·30	0·27	0·18	0·33
Lecithin %	10·9	11·4	7·3	6·5	4·5	8·0
Thymol %	35	32	31	32	30	32
Cholesterol % (× 1·4)	14·7	20·4	14·7	19·7	13·6	16·5
Recovery %	95	93	95	99	92	95

It appears from these figures that the precipitate is a protein-thymol-phospholipoid complex. 92 to 99 per cent. of its weight is accounted for if all the N is calculated as protein, the P as lecithin, and if the cholesterol is multiplied by the factor of 1·4, assuming it to be half esterified. The average composition is then protein 37·5 per cent., thymol 32 per cent., cholesterol 16·5 per cent., and lecithin 8·0 per cent. One unit of turbidity corresponded on the average to 106 mg. of precipitate per 100 ml. of serum, or to 40 mg. of protein, but there were large individual variations. It is for this reason that the units have been described as arbitrary.

It seemed probable that the protein would be the gamma globulin which is known to be concerned with other flocculation tests of liver function, and some support for this view was obtained by redissolving the wet precipitate in several cases in 0·01 N NaOH, when it was completely precipitated by one-third saturation with ammonium sulphate and by 15 per cent. sodium sulphite. This places it in the eu-globulin or high molecular weight fraction.

TABLE IV.—*Serum Proteins (g. per cent.) and Thymol Tests in 30 Cases.*

Number.	Thymol units.	Albumin. (Normal 4–5·5.)	Globulin. (Normal 1·5–3·0.)	Total. (Normal 6–8.)	Diagnosis.
1	24	2·4	7·8	10·2	Infective hepatitis.
2	21	3·3	3·6	6·9	,,
3	13	3·7	2·8	6·5	,,
4	12	4·3	2·2	6·5	,,
5	11	3·7	2·7	6·4	,,
6	10	2·9	3·0	5·9	,,
7	8	4·0	2·9	6·9	,,
8	1	3·9	2·2	6·1	,,
9	0	4·3	3·1	7·4	,,
10	28	1·8	5·6	7·4	Cirrhosis or subacute hepatitis
11	21	2·5	5·5	8·0	,,
12	14	3·9	2·3	6·2	,,
13	13	2·1	4·0	6·1	,,
14	9	2·3	4·5	6·8	,,
15	8	2·5	3·2	5·7	,,
16	6	2·7	3·3	6·0	,,
17	7	2·1	4·1	6·2	Haemochromatosis.
18	17	2·4	4·1	6·5	Lymphogranuloma.
19	6	3·4	2·2	5·6	Bacterial endocarditis.
20	10	1·7	2·6	4·3	,,
21	6	2·7	3·0	5·7	Rheumatoid arthritis.
22	3	4·2	3·3	7·5	,,
23	2	3·3	2·3	5·6	,,
24	2	3·8	2·7	6·5	,,
25	1	1·8	1·6	3·4	Nephritis.
26	4	1·8	2·0	3·8	,,
27	1	2·5	1·6	4·1	,,
28	0	4·6	2·2	6·8	,,
29	0	3·3	2·4	5·7	,,
30	2	3·6	3·2	6·8	Recurrent biliary obstruction.

Mechanism of Precipitation.

The chemistry of the precipitation is not understood, but it is suggested that the phenolic grouping has a special affinity for gamma globulin under the conditions employed, although other proteins are precipitated at more acid reactions or with higher concentrations of precipitant. Other protein precipitants lack this selective character, although there is a certain correlation with salting out procedures, as indicated by the serum globulin results given below.

Thymol Test and Serum Protein Concentration.

The results in Table IV were obtained by the manometric method of Peters and Van Slyke (1932, 2, 353, 691) using the selenate digestion mixture of Beaumont and Dodds (1941). The globulins were precipitated with 22·5 per cent. sodium sulphate.

The relationship shown here is very similar to that previously demonstrated between the serum proteins and the gold reaction (Maclagan, 1944*b*). There is a rough correlation with the serum globulin level, but about half the thymol-positive cases have normal globulins, and three cases (Nos. 9, 22 and 30) have negative thymol tests and raised globulins. The inversion of the albumin-globulin ratio in nephritis was associated with negative thymol tests. These findings are consistent with the view expressed above that the test depends upon a particular fraction of the globulin, and illustrates its special value in infective hepatitis, where the total serum globulin is frequently normal.

RESULTS.

The results in 248 cases in liver disease are given in Table V.

TABLE V.—*Thymol Tests in* 248 *Cases of Liver Disease.*

Diagnosis.	Number of cases.	Per cent. thymol positive.	Per cent. gold positive.	Thymol units. 0–4.	5–7.	8–10.	11–15.	16–20.	21–25.	26–30.	Means and st. errors.
Infective hepatitis	134	91	92	12	22	51	30	14	4	1	10·2 ±0·42
Cirrhosis	13	100	100	0	2	4	3	1	2	1	14·2 ±1·96
Arsenical	32	47	47	17	11	2	1	1	0	0	5·1 ±0·60
Weil's disease	23	52	30	11	9	3	0	0	0	0	5·1 ±0·46
Amyloidosis	7	14	29	6	1	0	0	0	0	0	1·7
Obstructive jaundice	39	7·7	7·7	36	3	0	0	0	0	0	1·2 ±0·27

It will be seen that the test is usually positive in infective hepatitis and in cirrhosis and is mainly negative in obstructive jaundice. No obstructive case had a value above 7 units, and 75 per cent. of those in the first two groups were above 7 units. In post-arsphenamine jaundice and in Weil's disease about half the results were positive, and amyloidosis gave mainly negative results. In infective hepatitis the turbidity was usually maximal at or soon after the onset of jaundice, and frequently remained positive for many weeks after the serum bilirubin became normal. Thus a typical case gave the following results at weekly intervals :

Thymol units.	Serum bilirubin.
12	10·0 mg.%
14	14·4 ,,
11	4·6 ,,
7	1·0 ,,
5	0·6 ,,

The controls are provided by sera from 217 patients suffering from diseases not primarily affecting the liver (Table VI).

TABLE VI.—*Thymol and Gold Tests in 217 Control Patients.*

Diagnosis.	Number of cases.	Number thymol-positive.	Thymol units.	Number gold-positive.	Strength of gold reaction (maximum, 5).
Glandular fever	4	4	16, 14, 13, 5	4	5, 5, 4, 1
Rheumatoid arthritis	8	3	6, 6, 6	7	5, 4, 4, 3, 3, 2, 1
Heart failure	13	3	6, 6, 5	5	3, 2, 2, 1, 1
Pernicious anaemia	8	3	7, 6, 6	5	5, 5, 1, 1, 1
Chronic osteomyelitis	2	2	7, 5	2	5, 1
Pulmonary tuberculosis	18	1	7	1	3
Nephritis	8	0	..	2	1, 1
Peptic ulcer	18	0	..	2	1, 1
Lymphogranuloma	1	1	17	1	5
Atypical pneumonia	1	1	8	1	2
Pyonephrosis	3	1	6	1	1
Cholecystitis	3	1	5	0	..
Volvulus	1	1	5	0	..
Normal pregnancy	12	0	..	0	..
Others	117	0	..	4	1, 1, 1, 1
Totals	217	21		35	

These were mostly taken at random from specimens undergoing routine analysis for other reasons, but there was some selection of cases of pulmonary tuberculosis, heart failure, and glandular fever. All common medical and surgical conditions are represented, the most numerous being listed separately in the table. There were 21 positive thymol tests made up as indicated, and it is evident that a proportion of patients suffering from heart failure, chronic infections, gross anaemias and the like will give weakly positive results although the proportion is smaller than with the colloidal gold test (see below). Glandular fever, lymphogranuloma and atypical pneumonia are in a different category, being virus infections which may have a special tendency to attack the liver. It seems reasonable to assume that all the positive reactors may have had some degree of liver damage and confirmatory tests were attempted in 8 of these cases: 4 had urobilinuria, one was jaundiced (glandular fever), one had a raised serum globulin (lymphogranuloma), and one had a galactose index of 190 (normal 0–160).

The uniformly negative thymol tests in the other 196 cases appears to form an adequate series of " normal " controls.

Serum Colloidal Gold Reaction and Thymol Test.

In general there was a close correlation between the two tests, the whole series of 465 cases giving 186 positive thymol and 195 positive gold tests, the latter being carried out by the method of Maclagan (1944*a*). The closest agreement is seen in infective hepatitis, post-arsphenamine jaundice and obstructive jaundice, in all of which the proportion of positive results was almost identical with the two tests (Table V). There was, however, some variation in individual cases, so that, e.g., 3 cases of infective hepatitis had positive thymol and negative gold tests, and 4 had positive gold and negative thymol tests. In this condition 95 per cent. of cases were positive with one or other test, as opposed to 91 per cent. with thymol alone and 92 per cent. with gold alone (Table VII).

There were three principal differences. The thymol test was more often positive than the gold test in Weil's disease—52 per cent. against 30 per cent. In cirrhosis

TABLE VII.—*Thymol and Gold Tests in* 134 *Cases of Infective Hepatitis.*

Thymol units.	Gold numbers (maximum, 5).						Totals.
	0.	1.	2.	3.	4.	5.	
0–4	8	3	0	1	0	0	12
5–7	3	3	4	4	2	6	22
8–10	0	3	0	7	17	24	51
11–15	0	0	1	3	4	22	30
16–20	0	0	2	0	1	11	14
21–25	0	0	0	0	0	4	4
26–30	0	0	0	0	0	1	1
Totals	11	9	7	15	24	68	134

the gold results—all but one 5+—were rather more striking than the thymol—all positive but six cases between 6 and 11 units. The gold test was more often positive in heart failure, chronic infections, anaemias, etc., where there were 16 positive thymol to 30 positive gold tests (Table VI). Rheumatoid arthritis—7 positive gold and 3 positive thymol tests out of 8 cases—is particularly prominent in this group.

There is therefore a certain advantage in doing both tests, which is technically convenient, as they use similar volumes of serum and reagent.

DISCUSSION.

The thymol turbidity test does not test any known function of the liver, and should be regarded as an indicator of disturbed liver metabolism rather than as a function test. The following evidence, although indirect, strongly suggests that it is related to the serum gamma globulin content, or to some closely similar globulin fraction: The protein in the precipitate is of the eu-globulin (high molecular weight) type. There is a strong correlation with the serum colloidal gold reaction which is known to depend mainly upon gamma globulin (Kabat *et al.*, 1943). The test is chemically and clinically related to the cephalin-cholesterol test, which is also concerned with gamma globulin (Kabat *et al.*, 1943). The predominance of positive results in infective hepatitis and cirrhosis follows the known increase of gamma globulin in these conditions (Gray and Barron, 1943). As the electrophoretic method is not yet available for clinical use, the desirability of having a simple means of demonstrating an excess of this protein in the serum needs no emphasis. The thymol test can claim certain advantages over the other procedures mentioned, as the reagent is easier to prepare and the reading of the results is free from subjective error.

As with the flocculation tests, there is at present no absolute proof that the protein responsible is actually produced by the liver, and its possible origin in the reticulo-endothelial system must be admitted, particularly in such conditions as lympho-granuloma and glandular fever. These considerations do not, however, impair the clinical usefulness of the test, which is largely empirical in basis. The distribution of results in the material submitted above indicates that the test is a valuable indicator of certain types of liver dysfunction, positive findings being particularly prominent in infective hepatitis and cirrhosis. It is difficult to explain the great preponderance of negative results (and the normal gamma globulin figures of Gray and Barron, 1943) in obstructive jaundice, where liver damage is frequently revealed by other tests. As with the gold test, the results are statistically quite different in infective hepatitis—91 per cent. positive, mean $10{\cdot}2 \pm 0{\cdot}42$—and in post-arsphenamine jaundice—47 per cent. positive, mean $5{\cdot}1 \pm 0{\cdot}60$.

The principles governing the interpretation of results in individual cases are almost identical with those recently discussed for the serum colloidal gold reaction (Maclagan, 1944*b*), except that the disturbing influence of chronic infections, heart failure, etc., are even less prominent with the thymol test. The distinction between obstructive and toxic and infective jaundice is about equally well made with the thymol test, and as with the gold test, it may profitably be combined with the serum alkaline phosphatase for this purpose. It is hoped to deal with this aspect in more detail elsewhere.

SUMMARY.

(1) When a saturated solution of thymol at pH 7·8 and ionic strength of 0·01 is added to serum from patients with parenchymatous liver disease, a turbidity or precipitate results in many cases. This finding has been developed into a test suitable for routine use, giving results on normal serum of 0 to 4 units.

(2) The evidence presented suggests that the test is chemically related to the Pandy and the cephalin-cholesterol tests, and that positive results indicate the presence of an increased serum gamma globulin content. The precipitate consists of a globulin-thymol-phospholipoid complex.

(3) The thymol test runs closely parallel to the serum colloidal gold reaction except in Weil's disease (more often positive) and in chronic infections (less often positive). The total serum globulin is above normal in about half the cases with positive thymol tests.

(4) Results in 248 patients with known liver disease and in 217 control patients indicate that the test is a valuable indicator of liver dysfunction, particularly in infective hepatitis and cirrhosis. Findings are negative or occasionally weakly positive in obstructive jaundice. They are significantly different in infective hepatitis (91 per cent. positive) and in post-arsphenamine jaundice (47 per cent. positive).

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Postscript.—While this paper was in the press two cases of Banti's disease with hepatic cirrhosis have given negative results with both gold and thymol tests. Serum bilirubin and alkaline phosphatase levels were also normal, but both patients had urobilinuria and a raised galactose index.

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THE OXYGEN-STABLE HAEMOLYSIN OF GROUP A HAEMOLYTIC STREPTOCOCCI (STREPTOLYSIN S).

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SINCE the discovery by Marmorek (1895) that culture filtrates of streptococci possessed haemolytic activity, much work on streptolysin has been done; a perusal of the literature, however, reveals many discrepancies and conflicting statements. Todd (1938) put forward the view that this confusion was due to the fact that streptococci may produce two distinct haemolysins, named by him Streptolysin O and Streptolysin S, which differ in many properties. Since this view is not universally accepted, we wish to summarize the evidence in favour of it derived from previous work, and additional evidence described in this paper.

Early work (e.g. Besredka, 1901; McLeod, 1912; McLeod and McNee, 1913) tressed (*a*) the extreme lability of streptolysin, (*b*) the fact that it could only be produced in media containing serum, and (*c*) the fact that it was not antigenic. The streptolysin studied by these workers, produced in media containing serum, may be referred to for the present as "serum-streptolysin."

Neill and Mallory (1926) were the first to study streptolysin produced in media not containing serum, and showed that this streptolysin was oxygen-labile; the haemolytic activity rapidly disappeared on standing in the presence of oxygen, but not if kept under anaerobic conditions. This inactivation by oxygen is reversible, and the haemolytic activity is fully restored by subsequent additions of reducing agents, such as $Na_2S_2O_4$.

Todd (1932) then found that this oxygen-labile haemolysin, produced in serum-free media, was antigenic. Rabbits and horses injected with this haemolysin developed antibodies which specifically neutralized it, but did not neutralize serum-streptolysin. It was also found that the spontaneous decrease in haemolytic activity of serum-streptolysin occurred equally rapidly in the presence or absence of oxygen, and was not restored by reducing agents such as $Na_2S_2O_4$.

Comparing the chemical properties of the two haemolysins, we find that serum-streptolysin is an exceedingly labile substance, rapidly losing haemolytic activity on standing; this inactivation is not due to oxidation and appears to be completely irreversible.

Streptolysin produced in serum-free media, on the other hand, also loses its haemolytic activity on standing, but only if exposed to oxygen; this change, moreover, is completely reversible by reducing agents. It is a stable substance, and may be kept for months at 0°, regaining its full haemolytic activity on addition of reducing agents.

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Herbert and Todd (1941) studied the biochemical properties of this haemolysin, and obtained it in highly purified form. The purified haemolysin is a heat-labile protein containing –S–S– linkages. It is haemolytically inactive, but is activated by substances such as cysteine, glutathione, thiolacetic acid, $Na_2S_2O_4$, etc., known to reduce –S–S– linkages to –SH groups.

Comparing the serological properties of the two haemolysins, we find that serum-streptolysin is generally considered not to be an antigen; serum-free streptolysin, on the other hand, is antigenic, but its antibodies do not neutralize serum-streptolysin.

All the above evidence suggests that serum-streptolysin and serum-free streptolysin are two completely different substances, and in 1938 Todd put forward this view, naming them " streptolysin S " and " streptolysin O " respectively. The possibility exists, however, that streptolysin S is merely streptolysin O " modified " in some way by the presence of serum that makes it resistant to oxidation and reduction and to neutralization by anti-streptolysin O. The addition of serum to streptolysin O, however, has none of these effects (Todd, 1933), so the further assumption must be made that streptococci growing in serum media produce streptolysin O in a " nascent " state, in which it can react or combine with some constituent of serum. This was the view originally taken by Todd (1932), and is still held by some workers to-day. Evidence against this view is the fact that streptococci grown in high-titred anti-streptolysin O serum produce as much streptolysin S as when grown in normal serum (Todd, 1933). The main evidence for it came from the discovery (Todd, 1933) that, contrary to previously accepted views, serum-streptolysin is antigenic. The antibodies produced by its injection, however, do not neutralize serum-streptolysin itself, but neutralize the haemolysin produced in serum-free media, streptolysin O. This has been confirmed by Abdalla and McLeod (1939).

This fact, however, could be interpreted in two ways. It might mean that serum-streptolysin is streptolysin O combined with some constituent of serum that impairs its combination with antibody but not its antigenicity; on the other hand, it might mean that there are two distinct streptolysins, O and S, the one antigenic and the other not, and that streptococci growing in serum media produce small amounts of streptolysin O as well as large amounts of streptolysin S. The presence of the streptolysin O would be masked in *in vitro* tests, but on injection into animals, would stimulate production of anti-streptolysin O.

The second interpretation, that there are two distinct streptolysins, and that serum-streptolysin contains both of them, appeared to be proven when Todd (1938) showed that specific antibodies to streptolysin S existed. These cannot be produced by immunization with serum-streptolysin itself, but are produced by immunization with living cultures of streptococci.

At this point it seems relevant to mention the discovery of Weld (1934) that a powerful " haemotoxin " can be obtained from living streptococci by extracting the centrifuged cocci with serum. The extracts have a very high haemolytic titre and are lethal to mice in small doses.

Todd (1938) showed that Weld's haemotoxin is essentially similar to the culture filtrates of streptococci grown in serum broth, and like these, appears to contain both streptolysin S and streptolysin O, the former predominating. Animals immunized with it produce anti-streptolysin O, but no antistreptolysin S. On the other hand, anti-streptolysin S produced by immunization with living streptococci neutralizes Weld haematoxin, while anti-streptolysin O does not. In these respects, and in its extreme lability, Weld's haemotoxin is exactly similar to serum-streptolysin.

The demonstration of two distinct antibodies would seem to complete the evidence for the existence of two distinct streptolysins; until recently, however, it was not

possible to obtain streptolysin S free from streptolysin O, although the reverse is easily possible.

In this paper certain strains of group A streptococci are described that do not appear to produce streptolysin O under any conditions, either *in vitro* growing in serum-free broth, or *in vivo* on injection of living cultures. They produce, however, strong streptolysin S when grown in serum broth, or extracted with serum by Weld's method. The serum-streptolysins of these strains are serologically pure streptolysin S, uncontaminated by streptolysin O, and unlike the serum-streptolysins of other strains, *do not stimulate the production of anti-streptolysin O when injected into rabbits.* This, we feel, affords the final proof that the production of anti-streptolysin O on injection of serum-streptolysin is not due to any antigenic properties of streptolysin S, but to small amounts of streptolysin O in the serum-streptolysin ; or in other words, that streptolysins O and S are two completely distinct haemolysins.

Colebrook *et al.* (1942) isolated thirteen Group A strains (of types 11, 12 and 25) which behave in exactly the opposite manner ; these strains all produce streptolysin O, but produce no streptolysin S. This is further strong evidence for the non-identity of the two haemolysins.

EXPERIMENTAL.

(1) *Immunological Properties of Serum-streptolysins from Different Strains of Streptococci.*

Todd (1939) observed that of 32 different Group A strains, two, " Blackmore " and " Lang," were exceptional in producing no streptolysin O whatever when grown in the serum-free medium of Todd and Hewitt (1932) ; nevertheless these strains produce an active streptolysin S when grown in serum broth, or on extracting the centrifuged cocci with serum. It has now been found that streptolysin S preparations from these strains do not stimulate the production of anti-streptolysin O when injected into rabbits.

Table I shows the effect of immunizing rabbits with streptolysin S (in the form of Weld haemotoxin) prepared from (*a*) strain " Blackmore " (Group A, Type 11), which is one of the two exceptional Group A strains producing no streptolysin O *in vitro*,

TABLE I.—*Immunization of Rabbits with Streptolysin S from Different Strains of Group A Streptococci.*

Strain " Richards " forms streptolysin O in glucose-$NaHCO_3$ broth.
Strain " Blackmore " forms no streptolysin O in glucose-$NaHCO_3$ broth.

Days.	Dose (ml.).	Rabbits immunized with Weld haemotoxin from :— Strain " Blackmore." A.S.O titre. R. 125.	R. 126.	Strain " Richards." A.S.O titre. R. 127.	R. 128.
0	..	<0·5	<0·5	<0·5	<0·5
11	1·0	..	..	..	..
12	2·0	..	..	..	..
15	2·0	..	..	..	..
16	4·0	..	..	..	..
23	4·0	..	..	..	..
24	4·0	..	..	..	..
35	..	<0·5	<0·5	30·0	50·0

Weld extracts prepared according to Todd (1938); m.h.d. = 0·005 ml. Injections subcutaneous. R. 125, etc., indicates number of rabbit. A.S.O titre = anti-streptolysin O titre in units/ml. of serum.

and (*b*) from strain " Richards " (Group A, type 3), which produces a strong streptolysin O *in vitro*. The first group of rabbits show no rise of anti-streptolysin O titre, the second group a very marked rise. This shows that the production of anti-streptolysin O on injection of serum streptolysins into rabbits is due to the streptolysin O, and not to the streptolysin S that they contain.

By the use of selected strains such as " Blackmore," it is now possible to obtain streptolysin S free from streptolysin O. These strains do not appear to be capable of producing streptolysin O under any circumstances, *in vitro* or *in vivo*. This can be shown by immunizing rabbits with living streptococci. This is a very sensitive test, as strains which produce only small amounts of streptolysin O *in vitro* produce a marked rise in anti-streptolysin O *in vivo*. Table II shows the immunization of rabbits with living cultures of strain 63 T (Group A, type 22) which forms streptolysin O *in vitro*, and strain " Blackmore " which does not. The results show that 63 T forms streptolysin O *in vivo* also, while " Blackmore " forms none.

Table III shows similar experiments with streptococci of other groups. Group C (human) strains and Group G strains form streptolysin O *in vitro*, and immunization with these strains causes a rise in anti-streptolysin O. On the other hand, Group F strains do not form streptolysin O *in vitro*, and immunization with living cultures causes no rise in anti-streptolysin O.

TABLE II.—*Immunization with Living Cultures of Group A Streptococci.*

Strain 63 T forms streptolysin O *in vitro*.
Strain " Blackmore " forms no streptolysin O *in vitro*.

	Rabbits immunized with—					
	Strain 63 T (Type 22).				Strain " Blackmore " (Type 11).	
Days.	Inoculum (ml.).		A.S.O. titre.		Inoculum (ml.).	A.S.O. titre.
	R. 85.	R. 86.	R. 85.	R. 86.	R. 87 and 88.	R. 87. R. 88.
0	0·2 (V.)	..	<1·0	<1·0	0·2 (V.)	<1·0 <1·0
7	0·4 „	..			0·4 „	
14	1·0 „	..			1·0 „	
21	2·0 „	2·0 (V.)			2·0 „	
28	4·0 „	4·0 „			4·0 „	
35	6·0 „	6·0 „			6·0 „	
42	8·0 „	8·0 „			8·0 „	
49	..	..	<1·0	<1·0	..	<1·0 <1·0
57	0·1 (L.C.)	0·1 (L.C.)			0·1 (L.C.)	
71	0·2 „	0·2 „			0·2 „	
79	0·3 „	0·3 „			..	
80	..	..			0·3 „	
85	0·4 „	0·4 „			0·4 „	
92	..	0·5 „			0·5 „	
99	..	0·6 „			0·6 „	
105	..	..		30·0	..	
107	..	0·7 „			0·7 „	
115	..	..	50·0		..	<1·0 <1·0

Two rabbits immunized with each strain. All doses intravenous. V. = vaccine ; L.C. = living culture. R. 85, etc., indicates number of rabbit. A.S.O titre = anti-streptolysin O titre in units/ml. of serum.

(2) *Haemolysis on Blood-agar Plates.*

Since the production of zones of haemolysis on blood-agar plates is such a commonly used diagnostic test in the study of streptococci, it is interesting to know which of the two haemolysins is responsible for this phenomenon. It has been possible to test this point by the use of the strains described in Section I, which produce streptolysin S only, and the strains of Colebrook *et al.* (1942), which produce streptolysin O only.

TABLE III.—*Immunization with Living Cultures of Groups C, F and G.*

Strain "Neill," Group C (human), forms streptolysin O *in vitro*.
Strain "Baylor," Group F, forms no streptolysin O *in vitro*.
Strain "Valente," Group G, forms streptolysin O *in vitro*.

Days.	Rabbits immunized with— Strain "Neill" (Group C). Inoculum (ml.). R. 92.	A.S.O titre. R. 92.	Strain "Baylor" (Group F). Inoculum. (ml.). R. 89 and 90.	A.S.O titre. R. 89.	A.S.O titre. R. 90.	Strain "Valente" (Group G). Inoculum (ml.). R. 94.	A.S.O. titre. R. 94.
0	..	..	0·2 (V.)	<1·0	<1·0	..	<1·0
7	..	..	0·4 „	..	..	0·2 (V.)	..
14	..	..	1·0 „	..	..	0·4 „	..
21	1·0 (V.)	<1·0	2·0 „	..	..	1·0 „	..
28	2·0 „	..	4·0 „	..	..	2·0 „	..
35	4·0 „	..	6·0 „	..	..	4·0 „	..
42	6·0 „	..	8·0 „	..	..	6·0 „	..
49	8·0 „	..	..	<1·0	<1·0	8·0 „	..
56	..	<1·0	..	..	..	..	<1·0
57	..	..	0·1 (L.C.)	..	..	..	..
71	0·1 (L.C.)	..	0·2 „	..	..	0·1 „	..
79	0·2 „	..	0·3 „	..	..	0·2 „	..
85	0·3 „	..	0·4 „	..	..	0·3 „	..
92	0·4 „	..	0·5 „	..	..	0·4 „	..
99	0·5 „	..	0·6 „	..	..	0·5 „	..
107	0·6 „	..	0·7 „	..	..	0·6 „	..
115	..	..	..	<1·0	<1·0	..	..
116	..	25·0	..	..	..	..	25·0
196	..	..	..	<1·0	<1·0	..	..

R. 92, etc., indicates number of rabbit. V. = vaccine; L.C. = living culture. All doses intravenous.
A.S.O titre = anti-streptolysin O titre in units/ml. of serum.

Confirming the results of Colebrook *et al.*, three strains supplied by them (one type 11 and two type 12) all produced streptolysin O in glucose-bicarbonate broth, but produced no trace of streptolysin S in serum broth. On horse blood-agar plates, however, they were apparently non-haemolytic, giving neither α- nor β-haemolysis, whether grown on the surface or in deep colonies, aerobically or anaerobically.

Since normal horse serum may contain considerable amounts of anti-streptolysin O, blood-agar plates were made with rabbit blood containing no detectable trace of anti-streptolysin O. On these plates surface colonies of the above strains, grown aerobically, showed no haemolysis, but deep colonies showed good β-haemolysis. Anaerobically, neither surface nor deep colonies were haemolytic.

The group A strains "Blackmore" and "Lang," however, which produce streptolysin S but no streptolysin O (section I), give zones of β-haemolysis on horse or rabbit blood-agar plates, both in surface and deep colonies, which are indistinguishable from the zones produced by "normal" Group A strains, which form both haemolysins. Moreover, addition of high-titred anti-streptolysin O serum to the blood-agar plates made no difference to the zones of haemolysis around surface colonies of strains that form streptolysin S.

These experiments indicate that haemolysis on the surface of blood-agar plates is entirely due to streptolysin S; streptolysin O is presumably oxidized under these conditions, so that strains such as those of Colebrook *et al.*, which produce this haemolysin only, appear non-haemolytic on surface plates.

At a lower oxygen-tension, in deep colonies, haemolysis may be due to streptolysin O, provided that the blood used contains no anti-streptolysin O, but under completely anaerobic conditions no streptolysin O is apparently produced.

(3) *Properties of Streptolysin S.*

(a) *Haemolysis.*

The haemolytic activity of streptolysin S preparations was determined by the accurate colorimetric method described elsewhere (Herbert, 1941). Dilutions were made in isotonic borate buffer of pH 8·0, since streptolysin S is most stable at this pH, and the time of incubation was 2 hours at 37°. The amount of haemolysin that

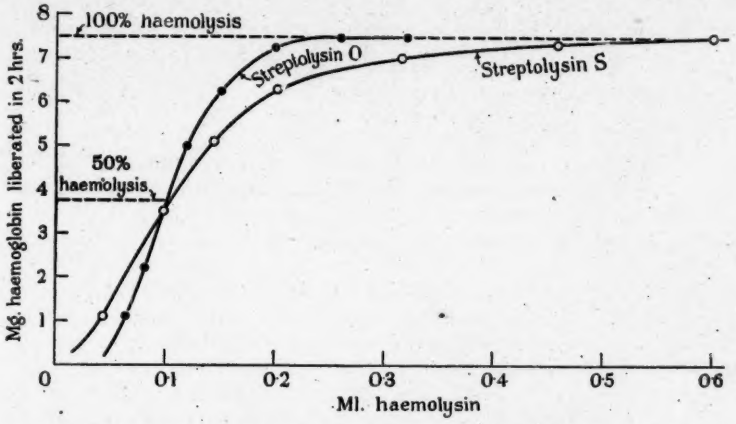

FIG. 1.—Concentration—per cent. haemolysis curves for streptolysins S and O.

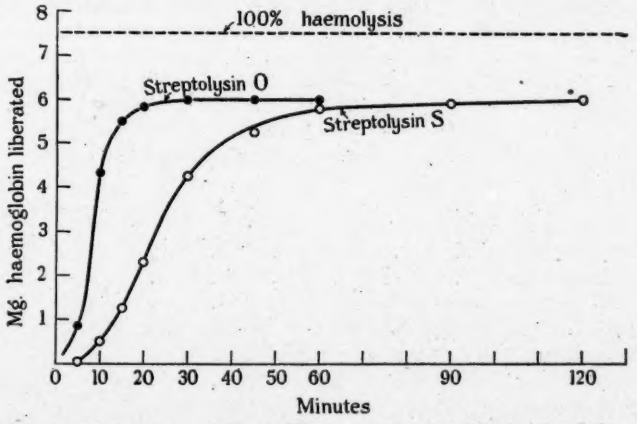

FIG. 2.—Time—per cent. haemolysis curves for streptolysins S and O.

under these conditions liberates 3·75 mg. haemoglobin from a suspension of rabbit's red cells containing 7·5 mg. haemoglobin, in a total volume of 2 ml., is called one haemolytic unit (H.U.).

Fig. 1 shows an experiment in which varying amounts of streptolysin S were incubated with red cells, and the degrees of haemolysis measured; a parallel experiment was run with a streptolysin O solution, diluted to contain the same number of H.U./ml. The marked difference in the shape of the two haemolysis curves is a constant feature of all preparations of the two haemolysins examined, and is unaffected

by purification. The theoretical basis of such haemolysis curves has been discussed previously (Herbert, 1941). These results indicate a difference in the haemolytic action of the two streptolysins.

Todd (1938) observed that streptolysin S was a " slower " haemolysin than streptolysin O. Fig. 2 is a quantitative demonstration of this difference. The amounts of the two haemolysins were adjusted to bring about the same ultimate degree of haemolysis (80 per cent.), but it will be observed that with streptolysin O all haemolytic action has ceased after *ca.* 15 minutes, while with streptolysin S haemolysis continues for a much longer period and is not complete for two hours. This also indicates a difference in the nature of the two haemolysins and their mode of action on the red cell.

(b) *General properties of streptolysin S.*

Streptolysin S, whether in the form of serum broth culture filtrates or Weld extracts, is very unstable. The haemolytic activity of fresh preparations falls to half its original value in approximately 10 hours at room temperature, 3 days at 0°, or 3 weeks at — 10°, though it can be preserved for a longer period at the temperature of solid CO_2. In contrast, streptolysin O may be kept for at least one month at 0°, or six months at — 10°, without loss of haemolytic activity (Herbert, 1941).

The inactivation of streptolysin S on standing is not due to oxidation, and appears to be irreversible. Loss of haemolytic activity is not prevented by storing anaerobically even in the presence of reducing agents, such as thiolacetate or cysteine, and is not restored by addition of such reducing agents ; neither is it accelerated by oxidizing agents such as H_2O_2. In all these respects the behaviour of streptolysin O is exactly the reverse (Table IV). The suggestion that streptolysin S is streptolysin O combined with some constituent of serum that renders it resistant to oxidation and reduction seems improbable ; attempts to increase the activity of active streptolysin S or to restore that of the partially inactivated material have been made, using varying concentrations of Na_2SO_3, $Na_2S_2O_4$, cysteine, thiolacetic acid and reduced glutathione, all without success.

TABLE IV.—*Effect of Oxidizing and Reducing Agents on Streptolysin S and Streptolysin O.*

	Haemolytic activities (H.U./ml.).	
	Streptolysin S.	Streptolysin O.
(1) Fresh culture filtrate	135	26
(2) Ditto + M/25 cysteine	130	202
(3) Ditto + M/25 cysteine followed by M/25 H_2O_2	132	0
(4) Culture filtrate after standing 2 days at room temperature	0	0
(5) After standing 2 days at room temperature ; M/25 cysteine added before testing	0	197
(6) M/25 cysteine added ; then stood 2 days at room temperature under anaërobic conditions	0	200

Streptolysin S is not inactivated by dialysis. Dialysed solutions may be dried *in vacuo* from the frozen state, forming a light brown powder, readily soluble in water. The dried material retains its full haemolytic activity over long periods, and freeze-drying is probably the best method of preservation.

(c) *Purification of streptolysin S.*

Twelve-hour culture filtrates of strain " Blackmore " (which produces streptolysin

S, but no streptolysin O) in 20 per cent. horse serum-broth were used as starting-material for purification ; the following are the details of a typical preparation.

1000 ml. culture filtrate (95 H.U./ml.) were dialyzed against distilled water overnight at 0°, and a small precipitate removed by centrifuging. CO_2 was passed through the supernatant (cooled in ice) for 15 min. and the resulting precipitate centrifuged down. (*N.B.*—The preliminary dialysis is essential, as CO_2 produces no precipitate if salts are present.) The supernatant, which contained no haemolysin, was discarded, and the precipitate dissolved in 100 ml. borate saline (pH 8) to give a distinctly opalescent solution containing 530 H.U./ml. (Solution I).

Saturated $(NH_4)_2SO_4$ solution, to which sufficient 0·880 ammonia had been added to bring the pH to 8, was then run into solution I, dropwise and with good stirring. Three successive precipitates were removed by centrifugation at increasing levels of $(NH_4)_2SO_4$ concentration, and dissolved in borate buffer, giving solutions I.a, I.b, and I.c. I.a was strongly opalescent, I.b was moderately opalescent, and I.c almost water-clear. Nearly all the haemolytic activity was in fraction I.a, which was refractionated with $(NH_4)_2SO_4$ as above.

Again three successive fractions were removed and the precipitates dissolved in borate giving solutions I.a(1), I.a(2) and I.a(3). The first was very strongly opalescent and contained the bulk of the haemolytic activity, the second was moderately opalescent and had a low haemolytic activity, and the third, which was almost water-clear, had no haemolytic activity. The yields and haemolytic activities of the various fractions are set out in Table V.

TABLE V.—*Purification of Streptolysin S.*

Stage.	Volume (ml.).	H.U./ml.	Total H.U.	Dry weight (mg./ml.).	H.U./mg. dry weight.	Total lipoid %.	Lipoid P.%.
Culture filtrate	1000	95	95,000	32·7	2·9	3·34	0·04
I. (CO_2 ppt.)	100	531	53,100	29·5	18·0	8·81	0·11
I.a. ($(NH_4)_2SO_4$ fractions)	53	415	22,000	12·4	33·5	14·3	0·23
I.b. ,, ,,	23	246	5,650	15·3	16·1	7·2	0·08
I.c. ,, ,,	23	44	1,100	20·9	2·1	0·5	0·01
I.a.(1) ,, ,,	33	324	10,700	6·24	52·0	20·5	0·34
I.a.(2) ,, ,,	30	188	5,640	6·47	29·0	12·6	0·20
I.a.(3) ,, ,,	32	36	1,150	5·1	7·2	4·4	0·06

Fraction I.a(1) represents the purest streptolysin S we have been able to obtain. It had 52 H.U./mg. dry weight compared with 2·9 H.U./mg. in the starting material. The increasing instability of the purified streptolysin S made further purification impossible.

The most striking feature of purified streptolysin S preparations was their strong opalescence, concentrated solutions almost resembling milk in appearance. They contained, however, no microscopically visible particles, and no material that could be separated by high-speed centrifugation (3 hours at 16,000 r.p.m.).

The purest fraction, after thorough dialysis, was dried and analysed for nitrogen (micro-Kjeldahl) and total phosphorus (method of Horecker, Ma, and Haas. 1940) and total carbohydrate by the orcin method (Pirie, 1936). Found: N, 12·1; P, 0·4; carbohydrate (as glucose), 0·1 per cent. It gave strong biuret reactions and colour reactions for tyrosine and tryptophane ; analysis by the method of Weber (1930) showed an arginine-content of 4·1 per cent. The material therefore consists mainly of protein, but the low N-content and high P-content suggest the presence of some other constituent, which cannot be a carbohydrate. The general appearance of the material suggested that it might be a lipo-protein ; all the fractions were therefore analysed for total lipoid by a modification of the method of Bloor (1928). 2 ml. samples of the

thoroughly dialyzed solutions were run into 100 ml. of an alcohol-ether mixture (3 : 1) containing 1 per cent. HCl, and boiled ; the filtrates were evaporated and the lipoid residue weighed. Lipoid-phosphorus was also determined in the alcohol-ether extracts ; both sets of results are shown in Table V.

There is a marked correlation between the values in columns 6, 7, and 8 of Table V ; the purer the streptolysin S, i.e. the higher the ratio of haemolytic activity to dry weight, the higher is the total lipoid and lipoid-P content. This suggests that streptolysin S either is, or is associated with, a lipo-protein.

(d) *Effect of organic solvents.*

The haemolytic activity of crude and purified streptolysin S preparations is completely destroyed by extraction with ether and ethyl acetate. After extraction, no haemolytic activity was found in either the aqueous layer or in the ether extract (after evaporating off the ether), or in a mixture of the two. The effects of these and other organic solvents (Table VI) run roughly parallel with their solubilities in water.

The same pure solvents had no effect on streptolysin O. (A sample of ether which contained peroxides did inhibit, but peroxide-free ether did not ; the effect is doubtless similar to that of H_2O_2.)

This effect of organic solvents seems to confirm the impression that streptolysin S may be associated with lipo-protein.

TABLE VI.—*Effect of Organic Solvents.*

Streptolysin S was a filtrate of a serum-broth culture of strain " Blackmore " ; streptolysin O was a purified preparation from strain " Richards." 10 ml. volumes extracted once with 10 ml. solvent. The ether was freed from peroxides, the ethyl acetate from free acids.

(1) *Streptolysin S.*

Solvent.	Solubility of solvent in water at 15° (vol. %).	Haemolytic activity (H.U./ml.).	Per cent. inhibition.
None	..	70	..
Petrol ether	0·005	68	3
Carbon tetrachloride	0·05	63	10
Benzene	0·082	42	40
Chloroform	0·42	40	40
Ethyl acetate	7·75	0	100
Ether	8·11	0	100

(2) *Streptolysin O.*

None	..	862	..
Petrol ether	..	860	<1
Chloroform	..	855	<1
Ether	..	863	<1

(e) *Weld's haemotoxin.*

Up to the present, streptolysin S has been obtained in high concentration only by growing streptococci in serum broth, or by extracting the organisms with serum as in the preparation of Weld's haemotoxin. Weld (1934) found that no streptolysin S is extracted by 0·9 per cent. NaCl, and suggested that the haemolytic substance is

TABLE VII.—*Haemolytic Activity of Streptococcal Extracts.*

Streptococci extracted with—	Volume of extract (ml.).											
	·5.	·2.	·1.	·05.	·02.	·01.	·005.	·002.	·001.	·0005.	·0002.	·0001.
(1) 0·9% NaCl	O	O	O	O	O	O	O	O	O			
0·9% NaCl—equal vol. serum added after extraction	O	O	O	O	O	O	O	O	O			
Serum diluted with equal vol. 0·9% NaCl	C	C	C	C	C	C	C?	++++	+++			
Serum conc. × 2 by ultrafiltration	C	C	C	C	C	C	C	C	C	++++	+++	++
Serum ultrafiltrate	O	O	O	O	O	O	O	O	O			
Normal horse serum	C	C	C	C	C	C	C	C	C?	++++	+++	+
(2) Lecithovitellin	C	C	C?	++++	++	tr.	O	O	O			
Lecithovitellin conc. × 4	C	C	C	C?	++++	++	tr.	O	O			
Normal human serum	C	C	C	C	C	C	C	C	C?	++++	tr.	O
(3) Crystalline egg albumin 4%	O	O	O	O	O	O	O	O	O			
Gelatin 2·5%	O	O	O	O	O	O	O	O	O			
Evans peptone 8%	O	O	O	O	O	O	O	O	O			
Difco proteose-peptone 8%	O	O	O	O	O	O	O	O	O			
Lecithovitellin	C	C	C	C?	++++	++	+	O	O			
Normal horse serum	C	C	C	C?	++++	+++	++	tr.	O			

Cocci from 500 ml. broth extracted with 8 ml. extracting fluid for 1 hour. Lecithovitellin prepared according to Macfarlane *et al.* (1941).
C = complete haemolysis; ++++, +++, ++, tr., = degrees of partial haemolysis.

only soluble in serum. We have made some experiments to test this hypothesis. The results, some of which are shown in Table VII, may be summarized as follows :

(1) If streptococci are extracted with 0·9 per cent. NaCl, the extract has no haemolytic activity. Phosphate buffers, Tyrode solution, etc., are likewise ineffective (*cf.* Schülter and Schmidt, 1936).

(2) If the organisms are extracted with saline and an equal amount of serum immediately added to the extract, the mixture has no haemolytic activity ; on the other hand, extraction with serum diluted with an equal volume of saline gives an extract of about half the haemolytic activity of an extract made with undiluted serum. Serum concentrated twice by ultra-filtration gives a more active extract than untreated serum. These experiments show (*a*) that the action of serum does not consist in conferring haemolytic properties on some haemolytically inactive substance which can be extracted by saline, but that serum must come into actual contact with the streptococci for any haemolysin to be extracted ; (*b*) the amount of haemolysin extracted is roughly proportional to the concentration of the serum.

These observations are in agreement with the " solubility " hypothesis. In further experiments attempts were made to fractionate normal horse serum, to determine which of its constituents are responsible for this effect.

Serum dialyzed two days gave Weld extracts of good haemolytic titre, while a serum ultrafiltrate was completely inactive, i.e. the crystalloidal constituents of serum are not concerned in this effect.

Serum from which lipoids had been removed by the method of Hartley (1925) (alcohol-ether at — 15°) extracted no haemolysin from streptococci ; ether-extracted serum extracted only one-tenth as much, and benzene and petrol ether-extracted serum one-half as much as untreated serum. Purified serum albumin, pseudo-globulin and euglobulin extracted little or no haemolysin (*cf.* Dyson, 1936).

These results suggested that the lipo-protein fraction of serum may be particularly concerned in the extraction. We therefore tried extracting streptococci with another lipo-protein, namely, crude lecithovitellin from egg-yolk. This extracted considerable amounts of streptolysin S (Table VII), though less than serum in most experiments. Other proteins and protein derivatives, e.g. crystalline egg albumin, gelatin, peptones, etc., did not extract a trace of haemolysin.

Lecitho-vitellin is the only substance yet found, other than serum, that will extract any considerable amount of streptolysin S from streptococci. The streptolysin S extracted by lecitho-vitellin is serologically identical with that extracted by serum ; it is neutralized by anti-streptolysin S, but not by anti-streptolysin O.

DISCUSSION.

The most striking characteristics of streptolysin S are (*a*) its intimate relation to serum, and (*b*) the fact that it is antigenic when part of the living streptococcal cell, but not when extracted from it.

In this connection it is important to stress the fact that streptolysin S is not formed *de novo* by the action of streptococci on serum, or *vice versa*, but is all the time present in the bacterial cells, from which serum has the power to extract it. This follows from the work of Smith (1937), who found washed streptococci suspended in saline haemolyzed added red cells, though no haemolysin could be extracted from the cocci by saline. This shows that the streptolysin S is present in the bacterial cell, and can exert its haemolytic effect if the red cells and streptococci are brought into intimate contact.

Streptolysin S resembles haptens such as the pneumococcal polysaccharides in

that, when extracted from the bacterial cells, it is not antigenic, but reacts with antisera made by immunizing animals with intact micro-organisms. One may tentatively conclude that it exists in the bacterial cell in rather labile combination with some protein that confers on it antigenic properties ; in the process of extracting the haemolysin from the cell this labile complex breaks down.

What the chemical nature of streptolysin S is, and why only serum and lecithovitellin are capable of extracting it from streptococci to any extent, is even more difficult to say with any certainty. The available facts suggest that this is a " solubility " effect, in which lipo-proteins play an important part. The term " solubility " is here used in a loose sense, and the effect may be not due to physical solution, but to the formation of some type of loose complex between the haemolysin and the lipoproteins of the extracting fluid.

The second object of this paper, namely, that of establishing whether or not streptolysin S and streptolysin O are the same haemolysin, we consider has been achieved. There seems no further doubt that these are two completely distinct haemolysins, and that different strains of Group A streptococci may produce either one or the other, or both together.

So dissimilar, in fact, are these haemolysins, that it is almost surprising that their non-identity should have been questioned. The main historical reason for this is the fact that immunization with streptolysin S prepared from most Group A streptococci gives rise to the production of anti-streptolysin O. We have now shown the reason for this, which is simply that the streptolysin S produced by such strains contains some streptolysin O, which is responsible for its antigenic effect. By choosing strains which produce no streptolysin O, streptolysin S preparations are obtained that produce no anti-streptolysin O on immunization, and which in fact produce no antibodies at all.

Some authors have stated, on purely *a priori* grounds, that it is unlikely for one organism to produce two haemolysins. It is now well established that *Cl. welchii* produces two distinct haemolysins, α and θ (Macfarlane *et al.*, 1941 ; Todd, 1941 ; van Heyningen, 1941) ; the same is probably true of *Cl. oedematiens* (Dr. C. L. Oakley, private communication), while at least three staphylococcal haemolysins are known to exist (Glenny and Stevens, 1936 ; Smith and Price, 1938*a*, *b*), and possibly more (Morgan and Graydon, 1936 ; Minett, 1936). The behaviour of haemolytic streptococci, therefore, is the rule rather than the exception.

SUMMARY.

(1) Certain strains of Group A streptococci are described which produce only streptolysin S ; other strains produce only streptolysin O. The majority of Group A strains produce both haemolysins.

(2) Haemolysis by surface colonies on blood-agar plates is entirely due to streptolysin S, and strains which only produce streptolysin O appear " non-haemolytic " by this test. Deep colonies may show haemolysis due to streptolysin O under certain conditions.

(3) Some chemical properties of streptolysin S and its partial purification are described. It is concluded that streptolysin S and streptolysin O are two entirely distinct haemolysins, both chemically and serologically.

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